REMARKS

Reconsideration of this Application is respectfully requested.

New claims 89-91 have been added and are supported throughout the instant specification. No new matter has been added.

Claims 1, 5, 6, and 83 have been canceled without prejudice.

Upon entry of the new and amended claims, Claims 2-4, 70-73, 75, 80, 82, 84, and 89-91 are pending in the application, with 70, 72, 73, 75, 80, 82, and 89-91 being the independent claims.

Based upon the foregoing Amendments and following Remarks, the applicants respectfully request the Examiner reconsider all outstanding objections and rejections, and that they be withdrawn.

Information Disclosure Statement

The Examiner asserts that a few references cited in the specification have not been listed in an IDS.

A supplemental IDS is herewith submitted citing references disclosed within the instant specification, but not previously submitted in an IDS.

Specification

The Examiner asserts that the nucleotide sequence disclosure contained in the instant application does not comply with the requirements for such a disclosure as set forth in 37 C.F.R. 1.821-1.825. Specifically, the Examiner asserts that the sequences in the specification do not have sequence identifiers.

Applicants herewith submit amendments to the specification that comply with 37 C.F.R. 1.821-1.825. No new matter has been added.

The specification has also been amended to correct minor typographical errors. No new matter has been added.

Rejection under 35 U.S.C. § 112, 2nd paragraph:

Claims 1-6, 70-73, 75, 80 and 82-84 stand rejected under 35 U.S.C. §112, 2nd paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention because the claims recite and encompass an isolated homozygous stem cell (claims 1-4) or a homozygous stem cell derived by a variety of methods (claims 5, 6, 70-73, 75, 80, 82-84), however the specification fails to clearly set forth what is considered "homozygous." Further, the Examiner asserts that it is unclear if the term "homozygous" reflects the clonal nature of the cell or to the genetic make-up and if it is in reference to the genetic make-up of a cell, the metes and bounds of the claims would be indefinite. The Examiner also asserts that upon review of the teachings of the specification it is unclear how one would identify or what would be considered "homozygous." According to the Examiner, "[e]ach individual comprises a set of chromosomes from both parents, and so at the least the combinations of any one of these would result in three possible combinations, for example in combining two spermatids (claim 5 and 6). It is unclear which of these three combinations would be considered homozygous."

The Examiner further asserts that claim 2-6, 70-73, 75, 80, and 82-84 indicate that the cells are "derived from," but what is encompassed by the term "derived" in how or how much the cells are altered is not clearly set forth in the claims nor the specification. Finally, the Examiner asserts that it is unclear how similar or different the resulting cell can be from the starting material and be considered encompassed by the metes and bounds of the claim.

Applicants respectfully traverse this rejection and request withdrawal of the rejection in view of the claim amendments and following remarks.

The term "homozygous" is described in the specification.

According to the specification on page 16, "'homozygous stem cell', previously termed a 'teratoma stem cell' or a 'TS cell', is an undifferentiated stem cell arising from a non-fertilized post-meiosis I diploid germ cell. Preferably, it is formed by preventing the extrusion of the second polar body during oogenesis (or 'activation'), or allowing the extrusion of the second polar body and spontaneous genomic self-replication of the haploid oocyte in appropriate conditions." The specification also provides on page 17 that the term "'homozygous post-meiosis I diploid germ cell'. . . means germ cells that are the stage of gametogenesis at which the cells contain two copies of either the paternal or maternal homologous chromosomes." Further, according to the specification on page 7 and throughout the application, a preferred embodiment of the invention is the Major Histocompatibility Complex (MHC); homozygous stem cells that contain two sets of identical MHC haplotypes.

It is also important to point out, and is well-known in the art, that the activated haploid replicates after the stage where potential for crossing over occurs (during meiosis I), which gives rise to identical copies resulting in homozygous stem cells. Further, the specification on page 6 states that "[u]pon proper activation, a metaphase II oocyte can proceed to complete meiosis by the extrusion of one of [sic] chromatid (i.e. the secondary polar body) and give rise to a haploid cell. Such meiosis-complete haploid oocyte self-replicates without cytokinesis, rendering it diploid and uniformly homozygous. Such meiosis-completed haploid oocytes, hence, may also be used to create the homozygous stem cells of the present invention with no heterozygosity." Specification, p. 6, last paragraph. The resulting stem cells would be homozygous for a particular haplotype because the activated haploid has already undergone any possible crossing over thereby giving rise to a diploid cell with two identical haploids. Therefore, the stem cells are homozygous because they contain identical homologous chromosomes, which are, i.e., homozygous for particular haplotypes, which is supported by the specification.

To further support the homozygosity of the claimed stem cells the following explanation is provided: The genome of an oocyte precursor is diploid (2N (P/M), where P and M are the chromosomes inherited by the father (P) and mother (M)). To develop into a mature oocyte DNA replication occurs with the genome becoming 4N (PP/MM). Next, cell division occurs rendering two daughter cells with the genome 2N (P/P) and 2N (M/M). The daughter cells would be homozygous 2N (P/P or M/M) because of the way the chromosomes segregate in this cell division. After cell division, one of the two daughter cells would condense and get extruded becoming the first polar body, i.e., 2N (P/P). The other daughter cell (2N (M/M)) would mature and become the oocyte. It is at this stage that activation would occur; naturally by a sperm or chemically as in the present invention. Natural fertilization would cause the 2N (M/M) oocyte to segregate into two 1N(M) and one would condense and become the second polar body. The other 1N(M) awaits the 1N from the sperm thereby creating a zygote which would be 2N. In the instant application, the second polar body can be extruded followed by self-replication or extrusion of the second polar body can be prevented. Therefore, the stem cells that develop from the chemical activation of the mature oocyte would be homozgyous (2N (P/P) or 2N (M/M).

The specification teaches how to identify cells that are homozygous.

The specification also teaches how one would identify or what would be considered "homozygous." Identifying homozygosity of cells is set forth in the specification at pages 62-63. For example, on page 62, the specification states, "[i]n order to reliably identify homozygosity in the limited amounts of DNA that were available after microdissection, multiple different microdissected tissue samples were analyzed with up to 14 distinct highly polymorphic microsatellite markers including DIS646 and DS243 (lp)..." Further, the claims have been amended such that preventing extrusion of the second polar body or extrusion of the second polar body followed by

self-replication are the methods used to create the homozygous stem cells. Fusing two oocytes or two spermatids or transferring nuclei have been removed, and as such the rejection no longer applies. The method discussed above can be used to determine and/or confirm the resulting stem cells are homozygous for particular haplotypes.

The claims have been amended to change the term "derived from" to "obtained from."

Finally, the claims that recite "derived from" are fully supported by the specification and the specification sets forth what is meant by the term. For example, claim 2 recites an isolated homozygous stem cell derived from human. Examples 1(b) and (c) set forth techniques for using human germ cells and parthenogenetically activating them to give rise to stem cells homozygous for, i.e., MHC haplotypes. Claim 3 recites an isolated homozygous stem cell derived from non-human species. Again, the specification provides support for the claim and claim terms. Example 1(a) sets forth a technique for using mouse post-meiosis I oocytes to generate, through parthenogenetic activation, homozygous stem cells. Mouse is one of the non-human species set forth in claim 4. Therefore, the term "derived from" is clearly set forth the instant application.

However, to expedite prosecution, the claims have been amended to recite "obtained from" instead of "derived from."

Applicants respectfully request reconsideration and withdrawal of these grounds of rejection in view of the amendment.

Additional support for the claimed invention.

To further support the instant application and illustrate that the methods of the instant specification work as disclosed, please find attached four references that followed the methods disclosed in the instant specification to successfully create homozygous stem cells (Cibelli *et al.*, Parthenogenetic Stem Cells in Nonhuman Primates, Science, Vol. 295, p. 819 (Feb. 2002); Vrana *et al.*, Nonhuman primate parthenogenetic stem cells,

PNAS, Vol. 100, Suppl. 1, pp 11911-11916 (Sept. 2003); and Mitalipov *et al.*, Parthenogenetic Activation of Rhesus Monkey Oocytes and Reconstructed Embryos, Biol. Reprod., Vol. 65, pp 253-259 (March 2001); Hwang *et al.*, Evidence of a Pluripotent Human Embryonic Stem Cell Line from a Cloned Blastocyst, Science, Vol. 303 (5664), pp. 1669-74 (Mar. 12, 2004; Epub. Feb. 12, 2004), Exhibits 1-4, respectively).

Rejection under 35 U.S.C. § 102 (b) – Thomson et al.:

Claims 1-6, 70-73, 75, 80, and 82-84 stand rejected under 35 U.S.C. §102(b) as being anticipated by Thomson *et al.* (Science 1998-IDS reference). The Examiner asserts that the above listed claims are generally drawn to an isolated homozygous stem cell and are produced by particular methods. The Examiner also asserts that the specification provides a definition of a "homozygous stem cell" as a cell previously termed a teratoma stem cell or a cell made by a variety of methods, including the isolation of stem cells isolated from the inner cell mass of blastocyst-like masses (see bottom of page 16). Finally, the Examiner asserts that the Thomson *et al.* reference teaches the isolation and characterization of human embryonic stem cells.

Applicants respectfully traverse this rejection. "A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." Verdegaal Bros. v. Union Oil Co. of California, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). Therefore, if a prior art reference does not teach each and every claim element, it does not anticipate the claim.

The Thomson *et al.* reference teaches embryonic stem cell lines derived from human blastocysts using human embryos produced by in vitro fertilization. In contrast, the present invention claims homozygous stem cells that are produced from no-embryonic or unfertilized stem cells. As such, the homozygous stem cells of the claims are a result of activation of post-meiosis I diploid germ cells. The instant invention does

not use oocytes fertilized by sperm cells. Further, the Thomson *et al.* reference does not teach the homozygosity of the resulting embryonic stem cells. Therefore, the Thomson *et al.* reference does not anticipate the pending amended claims because it does not teach each and every claim element, namely homozygosity.

Applicants respectfully request reconsideration of this rejection and withdrawal of these grounds of rejection in view of the aforementioned amendments and remarks. The pending claims are not anticipated by Thomson *et al.*

Rejection under 35 U.S.C. § 102 (b) – Doetschman et al.:

Claims 1-6, 70-73, 75, 80, and 82-84 stand rejected under 35 U.S.C. §102(b) as being anticipated by Doetschman *et al.* (J. Embryol. Morph 1985-IDS reference). The Examiner asserts that the above listed claims are generally drawn to an isolated homozygous stem cell and are produced by particular methods. The Examiner also asserts that the specification provides a definition of a "homozygous stem cell" as a cell previously termed a teratoma stem cell or a cell made by a variety of methods, including the isolation of stem cells isolated from the inner cell mass of blastocyst-like masses (see bottom of page 16). Finally, the Examiner asserts that the Doetschman *et al.* reference teaches the isolation and characterization of mouse embryonic stem cells.

Applicants respectfully traverse this rejection. The Doetschman *et al.* reference teaches the in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. However, the Doetschman *et al.* reference does not teach the homozygosity of the resulting mouse embryonic stem cells, nor parthenogenetically activated post-meiosis I diploid germ cells. Further, the embryonic stem cells disclosed by the reference are not pluripotent like the stem cells disclosed and claimed in the instant application. Therefore, the Doetschman *et al.* reference does not anticipate the pending amended claims because it does not teach each and every claim element, namely homozygosity.

Applicants respectfully request reconsideration and withdrawal of these grounds of rejection in view of the aforementioned amendments and remarks. Applicants submit that the pending claims are not anticipated.

Rejection under 35 U.S.C. § 102 (b) – Evans et al.:

Claims 1-6, 70-73, 75, 80, and 82-84 stand rejected under 35 U.S.C. §102(b) as being anticipated by Evans *et al.* (Nature 1981-IDS reference). The Examiner asserts that the above listed claims are generally drawn to an isolated homozygous stem cell and are produced by particular methods. The Examiner also asserts that the specification provides a definition of a "homozygous stem cell" as a cell previously termed a teratoma stem cell or a cell made by a variety of methods, including the isolation of stem cells isolated from the inner cell mass of blastocyst-like masses (see bottom of page 16). Finally, the Examiner asserts that the Evans *et al.* reference teaches the isolation and characterization of mouse embryonic stem cells.

Applicants respectfully traverse this rejection. The Evans *et al.* reference teaches the establishment in culture of pluripotential cells from mouse embryos derived from pregnant mice. However, the Evans *et al.* reference does not teach the homozygosity of the resulting cells, nor parthenogenetically activated post-meiosis I diploid germ cells. Therefore, the Evans *et al.* reference does not anticipate the pending amended claims because it does not teach each and every element of the claims, namely homozygosity.

Applicants respectfully request reconsideration and withdrawal of these grounds of rejection in view of the aforementioned amendments and remarks. Applicants submit that the pending claims are not anticipated.

Rejection under 35 U.S.C. § 102 (b) – Saito et al.:

Claims 1-6, 70-73, 75, 80, and 82-84 stand rejected under 35 U.S.C. §102(b) as being anticipated by Saito *et al.* (Dev. Biol. 1992-IDS reference). The Examiner asserts

that the above listed claims are generally drawn to an isolated homozygous stem cell and are produced by particular methods. The Examiner also asserts that the specification provides a definition of a "homozygous stem cell" as a cell previously termed a teratoma stem cell or a cell made by a variety of methods, including the isolation of stem cells isolated from the inner cell mass of blastocyst-like masses (see bottom of page 16). Finally, the Examiner asserts that the Saito *et al.* reference teaches the isolation and characterization of bovine embryonic stem cells.

Applicants respectfully traverse this rejection. The Saito *et al.* reference teaches bovine embryonic stem cell-like cell lines cultured over several passages that were derived from artificially inseminated superovulated cows. However, the Saito *et al.* reference does not teach the homozygosity of the resulting cells, nor parthenogenetically activated post-meiosis I diploid germ cells. Therefore, the Saito *et al.* reference does not anticipate the pending amended claims because it does not teach each and every element of the claims, namely homozygosity.

Applicants respectfully request reconsideration and withdrawal of these grounds of rejection in view of the aforementioned amendments and remarks. Applicants submit that the pending claims are not anticipated.

Double Patenting

Claims 1-6, 70-73, 75, 80, and 82-84 stand provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-5 and 7-14 of copending Application No. 10/179,959. The Examiner asserts that although the conflicting claims are not identical, they are not patentably distinct from each other because Application No. 10/179,959, a continuation of the instant application, have claims that set forth and are drawn to "homozygous stem cells" generated by the same methods as the instant application.

Applicants submit that the two applications are commonly owned and upon allowance of the claims, a terminal disclaimer will be filed.

CONCLUSION

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. It is believed that a full and complete response has been made to the outstanding Office action and, as such, the present application is in condition for allowance. Applicants wish to expedite the prosecution process and if the Examiner believes, for any reason that personal communication will help expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Response is respectfully requested.

Respectfully submitted,

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Nonhuman primate parthenogenetic stem cells

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Parthenogenesis is the biological phenomenon by which embryonic development is initiated without male contribution. Whereas parthenogenesis is a common mode of reproduction in lower organisms, the mammalian parthenote fails to produce a successful pregnancy. We herein describe *in vitro* parthenogenetic development of monkey (*Macaca fascicularis*) eggs to the blastocyst stage, and their use to create a pluripotent line of stem cells. These monkey stem cells (Cyno-1 cells) are positive for telomerase activity and are immunoreactive for alkaline phosphatase, octamer-binding transcription factor 4 (Oct-4), stage-specific embryonic antigen 4 (SSEA-4), tumor rejection antigen 1-60 (TRA 1-60), and tumor rejection antigen 1-81 (TRA 1-81) (traditional markers of human embryonic stem cells). They have a normal chromosome karyotype (40 + 2) and can be maintained *in vitro* in an undifferentiated state for extended periods of time. Cyno-1 cells can be differentiated *in vitro* into dopaminergic and serotonergic neurons, contractile cardiomyocyte-like cells, smooth muscle, ciliated epithelia, and adipocytes. When Cyno-1 cells were injected into severe combined immunodeficient mice, teratomas with derivatives from all three embryonic germ layers were obtained. When grown on fibronectin/laminin-coated plates and in neural progenitor medium, Cyno-1 cells assume a neural precursor phenotype (immunoreactive for nestin). However, these cells remain proliferative and express no functional ion channels. When transferred to differentiation conditions, the nestin-positive precursors assume neuronal and epithelial morphologies. Over time, these cells acquire electrophysiological characteristics of functional neurons (appearance of tetrodotoxin-sensitive, voltage-dependent sodium channels). These results suggest that stem cells derived from the parthenogenetically activated nonhuman primate egg provide a potential source for autologous cell therapy in the female and bypass the need for creating a competent embryo.

The use of human embryos to derive embryonic stem cells (ES cells) is viewed by some sectors of our society as ethically problematic. In nonhuman primates, there are currently three methods for deriving pluripotent stem cells: from embryos produced by *in vitro* fertilization (1–4), parthenogenesis (5), and from adult tissues such as cells derived from the bone marrow (6). We have previously reported the creation of a line of nonhuman primate stem cells from parthenogenetically activated eggs (5). By using this technique, ES cells were derived without the need to create or destroy a viable embryo.

Parthenogenesis, the process by which a single egg can develop without the presence of the male counterpart, is a common form of reproduction in nature. Flies, ants, lizards, snakes, fish, birds, reptiles, amphibians, honeybees, and crayfish routinely reproduce in this manner. Eutherians (placental mammals) are not capable of this form of reproduction. However, chimeras of parthenogenetic cells coupled with biparentally derived embryonic tissues have generated apparently normal offspring, and the parthenogenetic origin of several tissues has been confirmed in such chimeric animals (7). In a reported case of a human parthenogenetic chimera, contribution to several tissues has been demonstrated, including blood where 100% of the leukocytes were found to be of parthenogenetic origin (8).

Eutherian oocytes, on the other hand, can undergo parthenogenesis *in vitro* with variable success. When mammalian oocytes are activated (emulating the fertilization process) and transferred to a surrogate mother, they are capable of surviving to day 10 of development in the mouse, day 21 for sheep, day 29 in pigs, and day 11.5 in rabbit (9–12).

The reason for this halted development is believed to be due to genetic imprinting. It has been shown that maternal and paternal genomes are epigenetically different, and that both sets are required for successful development (13–15). In parthenotes (activated eggs), all of the genetic material is of maternal origin, and hence lacking paternal imprinting. It is believed that parthenotes are not capable of developing to term because they fail to develop a trophectoderm and primitive endoderm–extraembryonic tissues (9). They resemble ovarian teratomas and consist of only embryonic tissue. Androgenotes (created by the fusion of two sperm nuclei or diplodization of one sperm in the absence of female counterpart) are of purely paternal origin and develop into a structure consisting of a trophoblast and yolk sac (16). These resemble hydatidiform moles (solely trophoblastic tissue), which are formed when a sperm fertilizes an enucleated egg (17).

In the present report, we describe the parthenogenetic activation of cynomolgus macaque eggs *in vitro* and the derivation of a pluripotent cell line (Cyno-1). When cultured under selective conditions, these cells have divided for >2 yr, and, on induced differentiation, cell derivatives from all three germ layers were obtained.

Materials and Methods

Superovulation, Oocyte Retrieval, and Oocyte Maturation/Activation. Monkeys were injected (i.m.) with 1,000 units of pregnant mare serum gonadotrophin 5 days before surgery and then injected with 500 units of human chorionic gonadotropin 24 h before surgery. For ovary isolation, monkeys were tranquilized with ketamine (10 mg/kg of body weight), intubated endotracheally, and anesthetized with isoflurane (monitored to effect: no pal-

This paper results from the Arthur M. Sackler Colloquium of the National Academy of Sciences, "Regenerative Medicine," held October 18–22, 2002, at the Arnold and Mabel Beckman Center of the National Academies of Science and Engineering in Irvine, CA.

Abbreviations: ES cell, embryonic stem cell; Cyno-1 cell, parthenogenetically derived stem cell line from the cynomolgus macaque; bFGF, basic fibroblast growth factor; Snrpn, small nuclear ribonucleoprotein polypeptide N; TUJ1, β-tubulin III; PBL, peripheral blood lymphocytes; ICM, inner cell mass.

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pebral reflex, no deep pain response). Ovaries were removed by midline laparotomy incision.

Oocytes were manually harvested under a dissecting microscope. Oocyte maturation was performed in CMRL-1066 media (Sigma) with 20% FCS (HyClone), 10 units/ml pregnant mare serum (Sigma), 10 units/ml human chorionic gonadotropin (Sigma), 0.05 mg/ml penicillin, and 0.075 mg/ml streptomycin (Sigma). Eggs were incubated for 36 h at 37°C, in 5% CO_2 and 20% O_2. Mature metaphase II eggs were subsequently activated by incubation with 10 μM ionomycin for 8 min, followed by culture with 2 mM 6-dimethylaminopurine for 4 h. The inner cell masses (ICM) were isolated by immunosurgery as described (20) and cultured on a feeder layer of mitotically inactive mouse embryonic fibroblasts in Dulbecco's minimal essential medium (GIBCO) with 15% FCS (HyClone).

Cell Culture Conditions. Neural progenitor–ES cells were plated in flasks coated with fibronectin/laminin or fibronectin/BSA/collagen with NPMM (Clonetics, East Rutherford, NJ) and maintained at 37°C in 5% CO_2. Media were changed every 3 days. Differentiation was induced by removing basic fibroblast growth factor (bFGF) and epidermal growth factor, with the addition of 200 μM ascorbic acid.

Immunohistochemical Staining. A variety of markers of stem cells and stem cell differentiation were assessed by immunocytochemistry. Antibodies and staining conditions were as follows. For surface markers, cells were incubated with primary antibodies [stage-specific embryonic antigen-4 (1:20; Developmental Hybridoma Bank), tumor rejection antigen 1-81 (1:80), and tumor rejection antigen 1-60 (a gift from P. Andrews, Sheffield, U.K.)]. For immunocytochemisrty of embryonic markers, primary antibodies were diluted in PBS supplemented with 0.5% BSA. After washing with PBS-BSA, cells were fixed with 2% formaldehyde for 30 min and washed three times in PBS-BSA, followed by incubation with 10% normal goat serum in PBS at room temperature. Subsequently, primary antibody was added for 30 min at room temperature; cells were then washed with PBS-BSA three times, followed by incubation with secondary antibody for 30 min, washed, stained with 4′,6-diamidino-2-phenylindole (DAPI), and mounted. For immunocytochemistry of differentiated cells, cells were fixed in 4% paraformaldehyde at room temperature for 20 min, followed by permeabilization for 2 min in 100% ethanol. After fixation, cells were washed with PBS, blocked with 10% normal goat serum in PBS at room temperature for 2 h, followed by incubation at room temperature for 2 h with nestin antibody (1:200, Chemicon), TH polyclonal 1:200 (Pel-Freez Biologicals) or TH monoclonal 1:1000 (Sigma) β-tubulin type III (TuJ1) monoclonal (1:500, Babco, Richmond, CA), in PBS. After washing, cells were incubated with a rabbit secondary antibody in PBS-BSA at room temperature for 30 min. Cells were then washed in PBS and mounted.

Alkaline Phosphatase. Alkaline phosphatase was determined as described (5). Briefly, culture medium was removed from the plates, and cells were fixed with 4% paraformaldehyde for 20 min. Cells were washed three times in Tris-maleate buffer [(3.6 g of Trizma base (Sigma), in 1 liter of water, pH raised to 9.0 with 1 M maleic acid)] for 10 min each wash. The last wash was removed, and the staining solution [(Tris-maleate buffer: 200 μl of a 5 mM MgCl naphthol AS-MX phosphate (Sigma), 0.4 mg/ml; Fast red (Sigma), 1 mg/ml)] was added to the cells for 15 to 20 min. Once red colonies were detected, the reaction was stopped by adding PBS and bringing the pH to 7.4.

Antigen Profiling. Peripheral blood lymphocytes (PBLs) were isolated from whole blood by flotation on Ficoll-Hypaque. Cells were harvested from the medium:Ficoll-Hypaque interface, and the remaining red cells were hypotonically lysed. After additional washes, cells were labeled with FITC-conjugated anti-HLA-A,-B,-C [clone G46 52.6, Pharmingen) and phycoerythrin (PE)-conjugated anti-HLA-DR (clone G46.6, Pharmingen). Isotype-matched control antibodies were included as negative controls (shaded curves in Fig. 7). Cyno-1-derived neural cells were cultured, harvested, and stained with FITC-labeled anti-HLA-A,-B,-C and PE-labeled anti-HLA-DR as above and compared with cells stained with isotype-matched control antibodies (shaded curves in Fig. 7). Treatment of Cyno-1-derived neural cells with IFN-γ involved overnight incubation with human IFN-γ (40 ng/ml). Stained cells were analyzed with a FACScan flow cytometer and CELLQUEST software (Becton Dickinson).

Telomerase Activity Measurement. Telomerase activity was measured by using the TRAPeze kit (Intergen, Purchase, NY) as recommended by the manufacturer. Control template, buffer, and control extract were supplied by the TRAPeze kit. Extracts from the mouse feeder cells, the Cyno-1 cells (maintained on mouse feeder layer), and the differentiated Cyno-1 cells (grown for 14 days without mouse feeder layer) were normalized to the protein concentration. Heat inactivated extracts were boiled for 3 min before the assay.

Electrophysiology. The whole cell patch clamp technique was performed on differentiated stem cells that were continuously perfused with a Hepes-buffered saline (HBS) solution (containing, in mM: 150 NaCl, 10 Hepes, 2.5 KCl, 2.5 $CaCl_2$, 1.0 $MgCl_2$, 10 D-glucose, pH 7.4, with NaOH, osmolality 320 mmol/kg adjusted with sucrose). Tetrodotoxin (Calbiochem) was diluted from concentrated stocks into HBS and applied within 100 μm of the cell by using a linear array of fused-silica tubes (150 mm I.D., Hewlett–Packard) mounted on a manipulator. A Cs^+-based internal solution (in mM: 130 CsCl, 10 Hepes, 10 EGTA, 1 $CaCl_2$, 4 Mg-ATP, pH 7.2 with CsOH, osmolality 305 mmol/kg adjusted with sucrose) was used in the patch electrode.

Recordings were performed at room temperature according to published procedures by using an Axopatch ID amplifier (Axon Instruments, Foster City, CA) in voltage-clamp mode as described (18, 19). Whole-cell capacitance and series resistance were determined by fits of the capacitive transients during square-wave voltage steps by using standard software procedures contained within PCLAMP 7.0 software (Axon Instruments) and monitored throughout the recordings. Resting membrane potentials were −70 mV. Voltage-gated currents were elicited by square-wave membrane depolarizations to 0 mV.

Results

Creation and Characterization of Monkey Parthenogenetic Stem Cells. Stem cells were created via parthenogenetic activation of eggs as described (5). Briefly, 77 eggs were isolated from the ovaries of three different cynomolgus monkeys (*Macaca fascicularis*, ≈18 yr of age) after hormone-induced superovulation. The oocytes were then maintained in maturation medium for 36 h. Twenty-eight eggs reached metaphase II stage and were subsequently activated by incubation with 10 μM ionomycin for 8 min, followed by culture with 2 mM 6-dimethylaminopurine for 4 h. Four embryos developed to the blastocyst stage after 8 days in culture (14%) (Fig. 1*A*). Immunosurgically isolated ICMs (20) were cultured on a feeder layer of mitotically inactive mouse embryonic fibroblasts in Dulbecco's minimal essential medium with 15% FCS (HyClone). Three ICM showed outgrowth within 1 week of plating, and one stable cell line (Cyno-1) was successfully derived (Fig. 1*B*). Cyno-1 cells displayed many features that are typical for ES cells: cytoplasmic lipid bodies, small cytoplasmic/nuclear ratio, and clearly distinguishable nucleoli. These cells were immunoreactively positive for alkaline phosphatase, stage-specific embryonic antigen 4, tumor rejection antigen 1-60, and tumor rejection antigen 1-81 and were positive for octamer-

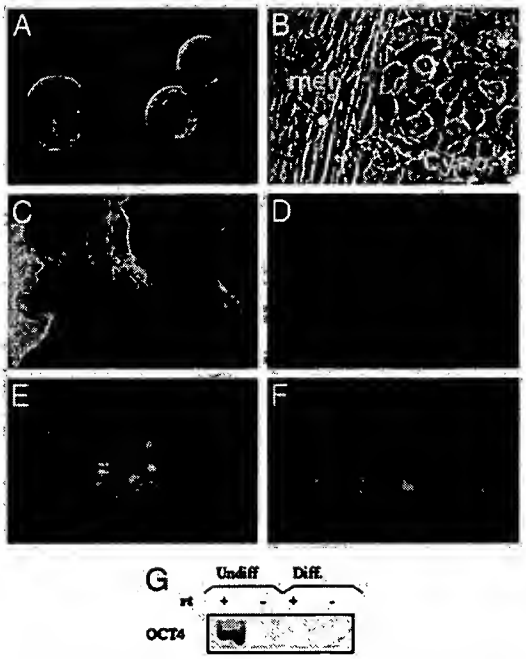

Fig. 1. Characterization of parthenogenetic embryos and derived cell lines. (*A*) Parthenogenetically activated eggs at day 8 of development before ICM isolation. (*B*) Phase contrast of Cyno-1 stem cells growing on top of mitotically inactivated mouse feeder layer (mef). (*C*) Alkaline phosphatase staining. (*D*) Stage-specific embryonic antigen 4. (*E*) Tumor rejection antigen 1-60. (*F*) Tumor rejection antigen 1-81 staining. (*G*) RT-PCR octamer-binding transcription factor 4 expression in undifferentiated Cyno-1 cells. (Scale bars = 50 μm in *A*, 10 μm in *B* and *D–F*, and 4 mm in *C*.)

binding transcription factor 4 mRNA (Fig. 1 *C–G*) and negative for stage-specific embryonic antigen-1 and -3 (data not shown). These cells have been propagated for >2 yr maintaining their undifferentiated state. Karyotype analysis revealed 40 + 2 chromosomes, in accordance with the species of origin, *M. fascicularis* (data not shown).

Parthenogenetic Stem Cell Differentiation. *In vitro* differentiation was induced by isolating the cells from the mouse feeder layer and culturing them in the presence of Dulbecco's minimal essential medium with 15% FCS; in some instances, 1,000 units of leukemia inhibitory factor was added to the media. A large variety of specialized cell types could be generated *in vitro*, such as spontaneously beating cardiomyocyte-like cells and ciliated epithelium, smooth muscle cells and cytokeratin-positive cells, as well as neuronal cells (data not shown).

To assess the differentiation capacity of Cyno-1 cells, we injected them into the peritoneal cavity of immunocompromised severe combined immunodeficient mice. Eight to 15 weeks after injection, teratomas were isolated and histologically analyzed. Microscopic observations revealed the presence of mature tissues and low frequency of mitotic figures, indicating their benign nature. Furthermore, derivatives of all three germ layers were observed, including cartilage, neurons, skin, and hair follicles (ectoderm), intestinal epithelia (endoderm), and muscle and bone (mesoderm) (Fig. 2).

Telomerase activity is often correlated with replicative immortality and is typically expressed in germ cells, cancer cells, and a variety of stem cells, including ES cells, but absent in most somatic cell types (21–23). Undifferentiated Cyno-1 cells displayed high levels of telomerase activity as detected by the TRAP assay (TRAPeze kit). However, no telomerase activity could be detected in differentiated progeny of Cyno-1 cells (Fig.

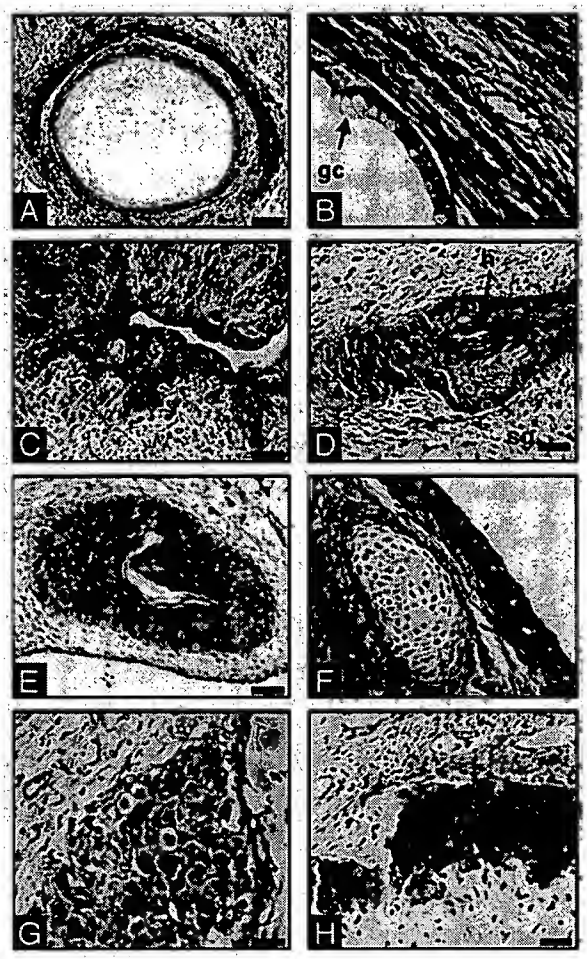

Fig. 2. *In vivo* differentiation of Cyno-1 cells. Cells were injected i.p. in severe combined immunodeficient mice. Eight and 15 weeks after injection, teratomas 12 and 30 mm in diameter, respectively, were isolated, fixed with 10% paraformaldehyde, and paraffin-embedded. Sections were stained with hematoxylin/eosin. The following complex structures were observed: gut (*A*), intestinal epithelium with typical goblet cells (gc) and smooth muscle (sm) (*B*), neuronal tissue with melanocytes (*C*), hair follicle complex with evident hair (h) and sebaceous gland (sg) (*D*), skin (*E*), cartilage (*F*), ganglion cells (*G*), and bone (*H*). (Scale bars = 40 μm in *A*, 10 μm in *B* and *D–H*, and 20 μm in *C*.)

3*A*). These data indicate a physiologically normal control of telomerase activity in Cyno-1 cells.

Genomic imprinting is initiated at gametogenesis and further modified during development. The small nuclear ribonucleoprotein polypeptide N (*Snrpn*) gene is an example of an imprinted gene that is expressed solely from the paternal allele (24, 25). It is monoallelically expressed from the onset of its expression at the four-cell stage (25). RT-PCR analysis confirmed the absence of *Snrpn* expression in Cyno-1 cells, whereas it is readily detected in heterozygous fibroblast cell cultures from the same species (Fig. 3*B*). Whereas biparental ES cells from *M. fascicularis* were unavailable to us for analysis, the *Snrpn* gene was readily detectable in biparental mouse ES cells under these same conditions (data not shown). These results indicate that the imprinting profile of at least one gene is consistent with the parthenogenetic origin of the Cyno-1 cells.

Nestin-Positive Neural Precursors. Cyno-1 ES cells were plated in flasks coated with fibronectin/laminin and NPMM (supplemented with bFGF, epidermal gowth factor, and neural survival

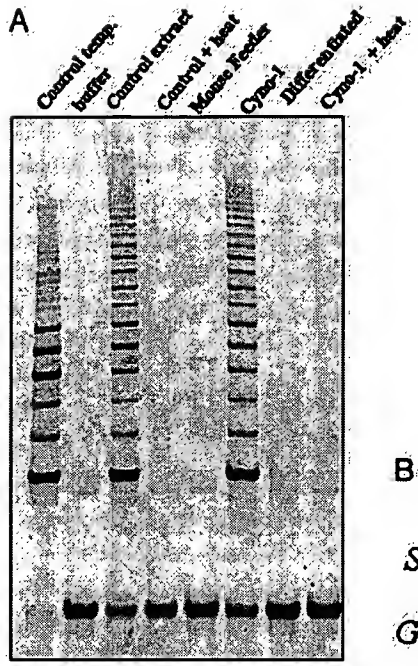

Fig. 3. Telomerase activity. (*A*) Cyno-1 cells, maintained in the undifferentiated state on mouse feeder layers, express telomerase activity that diminishes to undetectable levels in differentiated Cyno-1 cells. (*B*) RT-PCR to detect expression of the paternally expressed imprinted gene *Snrpn* in Cyno-1 cells (lane 1) and in adult fibroblasts (lane 2) from the same species. The housekeeping gene *Gapdh* is used as a control to demonstrate that equal amounts of mRNA were used.

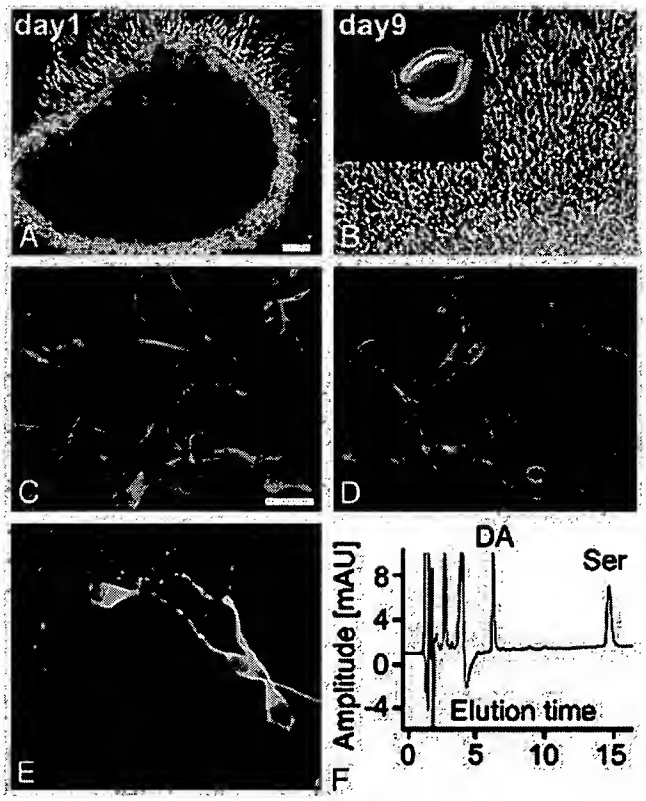

Fig. 5. Neural differentiation of Cyno ES cells *in vitro*. (*A*) Phase contrast microscopy of proliferating Cyno1-derived neural precursors at day 1 *in vitro* (DIV1). (*B*) Same cluster of precursors shown at DIV9. The total cell number has increased by 5- to 8-fold over a 9-day period. (*Inset*) One of many mitotic figures. Immunohistochemical analyses after 5 days of neural differentiation in the absence of bFGF and epidermal growth factor and the presence of ascorbic acid revealed positive staining for glial fibrillary acidic protein (GFAP), an astrocytic marker seen in *C*, and TUJ1, a neuronal marker seen in *D*. (*E*) Sequential exposure to sonic hedgehog, FGF8b, and ascorbic acid yielded an average of 25% TUJ1+ neurons coexpressing tyrosine-hydroxylase (TH), a marker for dopamine neurons. (*F*) HPLC revealing the release of dopamine (DA) and serotonin (Ser).

factor-1). These cells begin to differentiate into a neuronal-like morphology. Within 10 days, they do not differentiate further and proliferate at a rate of 5- to 8-fold increase over a 10-day period (Figs. 4 and 5 *A* and *B*). Their stage of development seems to be similar to those described by Ying *et al.* (26). Specifically, these cells express a high amount of nestin, which is an intermediate filament found in the developing CNS, mesenchymal tissue of the developing pancreas, and immature skeletal muscle (National Center for Biotechnology Information Locus Link).

With the removal of bFGF and epidermal growth factor and the addition of ascorbic acid, we are able to generate a high percentage of dopaminergic-like neurons (25% of TUJ+), glial, and epithelial cells. Immunocytochemistry stained positive for TUJ1, dopamine transporter (DAT), and microtubule associated protein-2, and negative for acetylcholine transferase, dopa-beta-hydroxylase, and NeuN (Fig. 5 *C–E* and data not shown). Cyno-1-derived neurons exhibited both basal and KCl-evoked synaptic release of dopamine and serotonin (Fig. 5*F*). Single-cell electrophysiology was also performed on these differentiated neurons. At around day 20, they begin to express voltage-

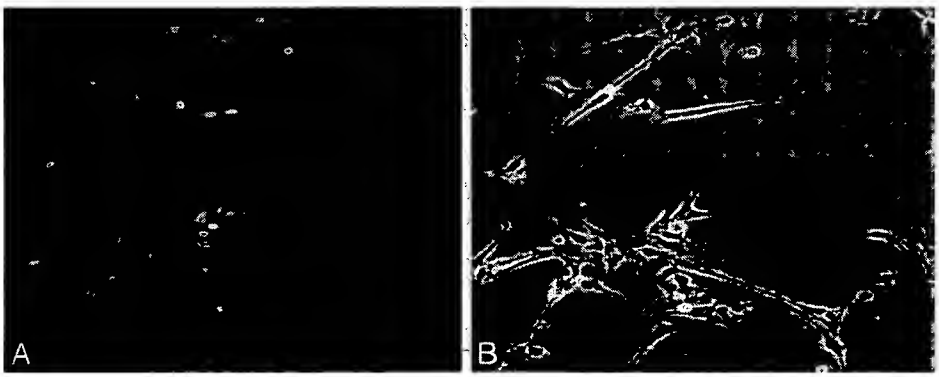

Fig. 4. Nestin-positive neural precursors derived from Cyno-1 cells. (*A*) Neural precursors stained for nestin (green). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). (*B*) Phase contrast of nestin precursors.

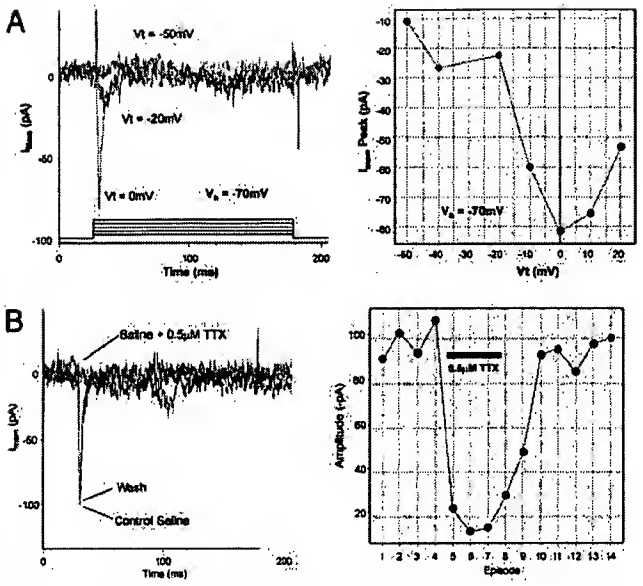

Fig. 6. Single-cell electrophysiology. (*A*) Neurons derived from Cyno-1 express voltage-dependent inward currents that are blocked by tetrodotoxin. Currents were elicited by membrane depolarizations to 0 mV every 15 s from a holding potential of −70 mV. Application of 0.5 μM tetrodotoxin inhibited >90% of these currents. (*B*) Inhibition was complete within 30 s of tetrodotoxin application and washed completely in <1 min.

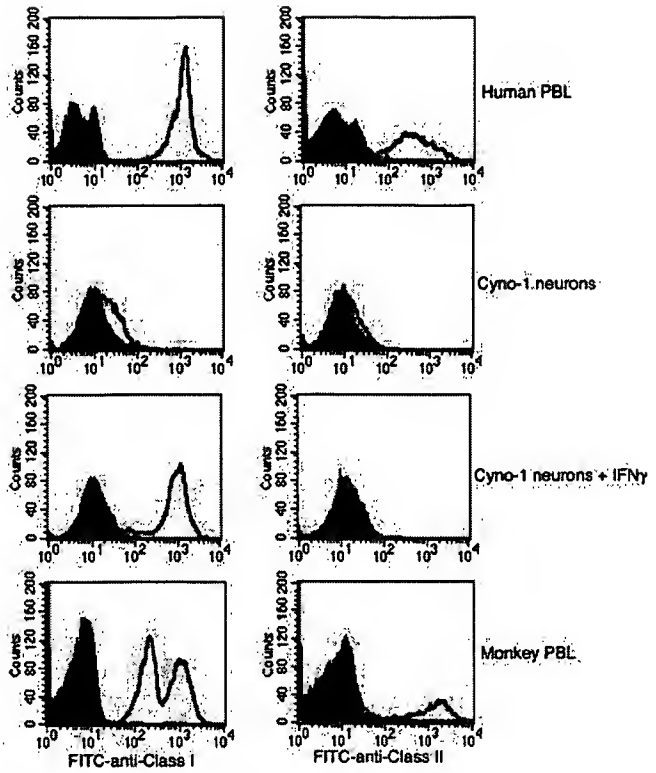

Fig. 7. Immunological profile of Cyno-1 cells. PBLs and Cyno-1-derived neural cells were analyzed by flow cytometry to quantitate expression of *M. fasicularis* class I (anti-HLA-A,-B,-C) and class II (anti-HLA-DR) antigens and compared with cells stained with isotype-matched control antibodies (shaded curves). PBLs express both class I and class II (DR) antigens whereas differentiated Cyno-1-derived neurons do not express either class of antigen unless treated with IFN-γ.

dependent sodium channels. The identities of these channels were confirmed by blocking with Tetrodotoxin. By day 30, 50% of neuron-like cells express these channels although nonc have yet to be identified in the neural precursors (Fig. 6). The differentiating cells assume morphological characteristics of neurons before functional ion channels become evident. This result suggests that there is a cascade of differentiation signals and/or precise coordination of differentiation events.

DNA Profiling of Cyno-1 Cells. To confirm their autologous origin, DNA profiling was performed. Total genomic DNA from the cynomolgus monkey oocyte donor (no. 5571) and from a preparation of cultured stem cells (Cyno-1; derived from no. 5571) were genotyped and compared by using seven simple sequence repeat (SSR) human markers (Research Genetics, Huntsville, AL) that had been shown previously to amplify monkey DNA and to discriminate between two individuals. The markers represent seven different chromosomes (nos. 3, 6, 7, 10, 11, 16, and 17) and in all cases except one (marker D16S403), alleles for no. 5571 were identical in number and size to the alleles for the Cyno-1 cells. An additional test was performed on DNA from no. 5571 and from the Cyno-1 cells (as well as two control animals). Micro SSPTM Generic HLA Class II DNA typing was performed in a 96-well tray format through the Wake Forest University–Baptist Medical Center Histocompatibility Laboratory. The data demonstrated that Cyno-1 stem cells and somatic cells from no. 5571 were indistinguishable from each other and therefore should be considered autologous (data not shown).

Histocompatibility Antigen Profile of Cyno-1. The histocompatibility antigen profile of Cyno-1-derived neurons was investigated and compared with lymphocytes from the oocyte donor by investigating polymorphic genes within the MHC that encode class I and class II cell surface proteins. These proteins present immunogenic peptides to $CD8^+$ and $CD4^+$ T cells, respectively. We have analyzed the Cyno-1-derived neural cells by flow cytometry for the expression of Mafa (MHC of *M. fascicularis*) class I and class II antigens. PBLs from the original cell donor expressed class I and class II antigens detected by antibodies specific for monomorphic HLA-A,-B,-C and HLA-DR antigens, respectively (Fig. 7). Seventy-five percent of PBLs were positive for class I, and 14% of PBLs were positive for class II. However, Cyno-1-derived neural cells were negative for both Mafa class I and class II antigens (Fig. 7), consistent with observations that these CNS cell types are class I- and class II-negative in normal murine CNS (27, 28). Viral infection or treatment with IFN-γ stimulates up-regulation of class I and class II expression in murine CNS cells. The ability of IFN-γ to up-regulate class I and class II expression by Cyno-1-derived neural precursor cells was investigated by preculturing these cells with IFN-γ (40 ng/ml) overnight before staining and flow cytometry. As shown in Fig. 7, pretreatment of Cyno-1 derived cells resulted in class I-specific staining with an intensity that was comparable to staining of normal human PBLs. However, IFN-γ treatment did not increase class II expression. These results support the prediction that an *in vivo* inflammatory response, expectedly involving IFN-γ expression, would up-regulate class I expression on transplanted Cyno-1-derived neural cells. Accordingly, in the event of transplantation into a nonisogenic animal, these cells should not escape surveillance by $CD8^+$ cytotoxic T lymphocytes.

Discussion

We have generated a primate parthenogenetic cell line (Cyno-1) with ES cell-like properties that can be propagated *in vitro* in an undifferentiated state for at least 2 yr. These cells express telom-

erase activity consistent with their extended lifespan property. The *in vitro* derivation of large numbers of specific cell lineages from Cyno-1 cells, including the generation of unlimited numbers of dopaminergic neurons, is of particular interest. In the present context, we have demonstrated that these cells (*i*) express TH, (*ii*) release neurotransmitter (dopamine and serotonin), and (*iii*) are electrophysiologically active. For these reasons, we believe that neurons, differentiated from parthenogenetic stem cells, may provide an important source of therapeutic treatments. Clinical transplantation of specific fetal neurons has shown promise in the treatment of Parkinson's disease (29) and Huntington's disease (30), but obtaining such cells from animals or human fetal brain remains problematic. Neurons derived *in vitro* from a renewable source, such as CNS precursors (31), ES cells (32, 33), or stem cells of parthenogenetic origin, could alleviate some of the ethical and technical concerns of human cell therapy.

Although the Cyno-1 parthenogenetic stem cell seems, in all respects, to be similar to traditional ES cells, it is reasonable to question their viability and utility. They are, after all, exclusively derived from maternal DNA. When trying to understand how these parthenogenetic stem cells are capable of developing into functional tissue, it is important to remember the following characteristics of genetic imprinting. First, ES cells are isolated from the blastocyst stage, this stage exhibits low DNA methylation levels, and the effects of imprinting could be minimized (34). Second, imprinting, in some cases, has been shown to not be completely silent: there are reports of mRNA expressed from imprinted genes that should not have been transcribed (24). Third, Surani and Barton (9) suggest that parthenogenetic embryos do not develop to term because of a high frequency of errors in X chromosome inactivation that occurs in extraembryonic tissues when both X chromosomes are derived only from the female. One might conclude that the effects of imprinting have a significant effect on extraembryonic tissue, and not the ICM from which our stem cells are derived.

One could speculate that these parthenogenetically derived stem cells are capable of differentiating into a high percentage of electrophysiologically active dopaminergic neurons due to the effects of genetic imprinting. It is believed, for example, that 0.1–1% of all mammalian genes are imprinted (35). However, only ≈50 imprinted genes have been identified in mice, some of which are conserved in humans (36). One of the most interesting characteristics of imprinting is that it occurs in clusters. The imprinting cluster on mouse chromosome 7 and the corresponding human chromosome 11p15.5 contains 14 imprinted genes (37). Tyrosine hydroxylase (TH), the rate-limiting enzyme of dopamine synthesis (38), is located on chromosome 11p15.5 and resides in the middle of a very well-characterized imprinted region that spans 1 Mb. There are in this cluster some of the best characterized imprinted genes, such as H19, insulin growth factor 2, and insulin growth factor antisense (37). Whereas tyrosine hydroxylase is biallelically expressed in mice, its imprinting status in humans has yet to be determined (37). Could this high expression level of tyrosine hydroxylase (in maternally derived parthenote cells) suggest it as an imprinted gene in nonhuman primates? Moreover, are these epigenetic modifications associated with imprinting permitting the robust expression of TH in Cyno-1-derived neural cells?

Further data analysis of functional genomic studies at the neural precursor and differentiated stage show normal gene expression of housekeeping genes such as the mRNAs of the 60S ribosomal subunit, the glycolytic pathway, and the tricarboxylic acid (TCA) cycle. Most interesting, when the "Stemness" genes recently described by Ramalho-Santos *et al.* (39) were converted into their human orthologs, 96 of 216 were expressed in the parthenogenetically derived monkey neural precursor cells (J.D.H., J. C. Mychaleckyj, and K.E.V., unpublished results).

We report here the isolation and further characterization of nonhuman primate parthenogenetic stem cells. These cells may provide a novel tool for assessing the effects of genomic imprinting on cell differentiation and function during development in primates. Their striking differentiation capabilities (electrophysiologically active, dopamine-secreting neurons) indicate their therapeutic potential and suggest a valid alternative to biparentally derived ES cells.

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Exhibit 2

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DEVELOPMENT

Parthenogenetic Stem Cells in Nonhuman Primates

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Parthenogenesis is the process by which an egg can develop into an embryo in the absence of sperm. This process has been characterized to some extent in nonhuman primates (*1*, *2*); however, to date, no primate parthenogenetic embryonic stem (ES) cell lines have been derived. Although attempts to obtain full-term mouse and bovine parthenogenetic individuals have failed (*3*–*5*), chimeras of parthenogenetic cells with biparentally derived embryonic tissues have generated apparently normal offspring (*6*).

Here we demonstrate broad differentiation capabilities of primate pluripotent stem cells derived by parthenogenesis. After in vitro maturation for 36 hours [with media supplemented with pregnant mare serum (10 IU/ml) and hCG (10 IU/ml); Sigma], 28 out of 77 primate eggs (*Macaca fascicularis*) reached metaphase II. Eggs were parthenogenetically activated with protocols previously described (*2*). Four out of 28 eggs (14%) developed to the blastocyst stage (Fig. 1A). Inner cell masses (ICM) were isolated by immunosurgery, and 1 week after plating, cell proliferation was observed in three ICMs and one stable cell line (Cyno-1) was obtained. Morphologically, the cells had a small cytoplasmic/nuclear ratio and numerous and prominent nucleoli and cytoplasmic lipid bodies. These cells could be extensively propagated in vitro (10 months) while maintaining their undifferentiated state. They tested positive for markers of primate ES cells with the exception of SSEA-3. High telomerase activity seen in the undifferentiated cells was lost completely after 2 weeks of differentiation, suggesting that telomerase activity is regulated in Cyno-1 cells and is not constitutive, as is observed in many tumor cell lines. In addition, karyotyping revealed 40+2 chromosomes, as is expected for *Macaca fascicularis*. Typing for sequence repeats and micro SSPTM generic human lymphocyte antigen class II DNA performed in Cyno-1 cells and somatic cells from donor animal were indistinguishable and therefore should be considered autologous.

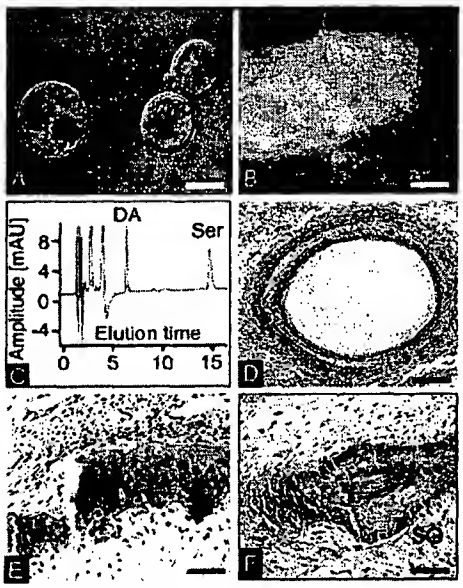

Fig. 1. Characterization of primate parthenogenetic embryos and derived cell lines. (**A**) Parthenogenetically activated eggs at day 8 of development before ICM isolation. (**B**) Upon induced differentiation, up to 25% of TUJ1$^+$ cells coexpressed tyrosine-hydroxylase (rabbit antibody to TH 1:250; PelFreez) exhibiting complex neurites. (**C**) Neuronal function was assessed by reverse-phase HPLC with electrochemical detection of the neurotransmitters dopamine (DA) and serotonin (Ser). (**D to F**) In vivo differentiation-teratoma. (D) Gut, (E) bone, and (F) hair follicle complex with hair (H) and sebaceous gland (SG). Scale bars: (A), 100 μm; (B), (E), and (F), 20 μm; (D) 50 μm.

Neural differentiation of Cyno-1 cells was induced with a multistep culture procedure (*7*), and astrocytes and neurons were obtained. Up to 25% of dopaminergic neurons could be obtained as judged by immunocytochemical criteria (Fig. 1B). Neuronal identity and function were confirmed by high-performance liquid chromatography (HPLC) analysis, which showed in vitro release of the neurotransmitters dopamine and serotonin (Fig. 1C). By modifying culture conditions, a large variety of specialized cell types could be generated in vitro, including spontaneously beating cardiomyocyte-like cells, smooth muscle cells, adipocytes, and beating ciliated epithelium, among others.

The capacity of Cyno-1 cells to differentiate was also tested in vivo by injecting them into the peritoneal cavity of immunocompromised severe combined immunodeficiency disease (SCID) mice (C.B-17 SCID; Charles River). Teratomas were isolated 8 and 15 weeks after injection and subjected to histological analysis. Derivatives of all three germ layers were observed, including cartilage, muscle and bone (mesoderm), neurons, melanocytes, skin and hair follicles (ectoderm), and intestinal and respiratory epithelia (endoderm) (Fig. 1, D to F). The presence of mature tissues and low frequency of mitotic figures in these tumors indicated their benign nature.

The in vitro differentiation of these cells to well-characterized dopaminergic neurons is of particular interest, because of their potential to replace lost neurons in Parkinson's disease. Thus far, dopaminergic neurons have been derived from mouse ES cells in vitro (*7*) but not from primate ES cells.

The proposal of human therapeutic cloning (HTC) describes the generation of autologous ES cells through somatic cell nuclear transfer (*8*). This study suggests an alternative to HTC. Differentiated cell types derived in vitro by parthenogenesis eliminate the requirement to produce or disaggregate a normal, competent embryo and may circumvent the ethical concerns voiced by some, positively impacting the debate in stem cell research.

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Exhibit 3

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Parthenogenetic Activation of Rhesus Monkey Oocytes and Reconstructed Embryos[1]

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ABSTRACT

This study determines the efficiency of sequential calcium treatments (electroporation or ionomycin) combined with protein synthesis (cycloheximide) or phosphorylation inhibitors (6-dimethylaminopurine) or the specific maturation promoting factor (MPF) inhibitor, roscovitine, in inducing artificial activation and development of rhesus macaque parthenotes or nuclear transfer embryos. Exposure of oocytes arrested at metaphase II (MII) to ionomycin followed by 6-dimethylaminopurine or to electroporation followed by cycloheximide and cytochalasin B induced pronuclear formation and development to the blastocyst stage at a rate similar to control embryos produced by intracytoplasmic sperm injection. Parthenotes did not complete meiosis or extrude a second polar body, consistent with their presumed diploid status. In contrast, oocytes treated sequentially with ionomycin and roscovitine extruded the second polar body and formed a pronucleus at a rate higher than that observed in controls. Following reconstruction by nuclear transfer, activation with ionomycin/6-dimethylaminopurine resulted in embryos that contained a single pronucleus and no polar bodies. All nuclear transfer embryos activated with ionomycin/roscovitine contained one large pronucleus. However, a third of these embryos emitted one or two polar bodies, clearly containing chromatin material. In summary, we have identified simple yet effective methods of oocyte or cytoplast activation in the monkey, ionomycin/6-dimethylaminopurine, electroporation/cycloheximide/cytochalasin B, and ionomycin/roscovitine, which are applicable to parthenote or nuclear transfer embryo production.

calcium, fertilization, IVF/ART, kinases, meiosis

INTRODUCTION

The production of genetically identical mammals by somatic cell nuclear transfer (NT) is now a reality [1–5]. However, low efficiencies and high embryonic, fetal, and neonatal losses necessitate further research into the basic mechanisms controlling the onset and early preimplantation development of embryos produced by NT. Factors known to affect the successful development of reconstructed embryos include the source of donor nuclei, the cell cycle stage of both the donor nucleus and recipient enucleated oocyte (cytoplast), and cytoplast activation. The latter, cytoplast activation, is an essential component of the NT procedure, because the introduction of the donor nucleus into the cytoplast bypasses fertilization.

During fertilization, sperm entry triggers a series of intracellular calcium oscillations critical to oocyte activation. Maturation promoting factor (MPF) and mitogen-activated protein (MAP) kinase are the most likely targets of calcium-stimulated events because inactivation of these kinases is a prerequisite to the resumption and completion of meiosis, subsequent pronuclear formation, and DNA synthesis [6–8]. MPF is a complex of two subunits: a catalytic subunit, $p34^{cdc2}$, a homologue of the yeast cdc2 protein kinase; and a regulatory subunit, cyclin B. Association of these subunits and subsequent activation of MPF occurs in a specific order by dephosphorylation of the $p34^{cdc2}$ subunit at threonine-14 and tyrosine-15, and by phosphorylation at threonine-161. MPF displays its peak activity at metaphase of mitotic cell cycles in association with nuclear envelope breakdown, chromatin condensation, and the formation of a mitotic spindle [9]. MPF inactivation, which is necessary for the cell to exit mitosis, involves cyclin proteolysis by the proteosome system [10].

In vertebrates, mature oocytes are arrested at metaphase of the second meiotic division (MII) with elevated MPF activity maintained by a cytostatic factor (CSF), essential components of which are the product of the c-*mos* proto-oncogene, MAP kinase, and possibly Cdk2 kinase. CSF prevents ubiquitin-dependant degradation of cyclin B and, thus, inactivation of MPF. Intracellular Ca^{2+} oscillations triggered by sperm down-regulate CSF activity and release the cyclin degradation machinery. Proteolytic degradation of cyclin B and subsequent MPF inactivation releases oocytes from metaphase arrest and allows the beginning or resumption of mitotic cycles [11].

Various artificial activation treatments mimic sperm-triggered events and induce parthenogenetic development in MII oocytes. For example, ethanol, electroporation, calcium ionophore, ionomycin, or inositol 1,4,5-trisphosphate induce calcium elevations and release meiotic arrest [12–17]. However, MPF activity, at least in young bovine and rabbit oocytes, is quickly restored with recondensation of chromosomes and reentry of activated oocytes into a new M-phase arrest, known as metaphase III [14, 18]. This phase can be circumvented by additional treatments that inhibit protein synthesis (cycloheximide, CHX) or protein phosphorylation (6-dimethylaminopurine, DMAP) [14, 16]. Thus, sequential approaches have evolved with ionomycin/DMAP, calcium ionophore/DMAP, calcium ionophore/CHX, or inositol 1,4,5-trisphosphate/DMAP that result in high activation and parthenogenetic development rates in the bovine and rabbit [14, 16, 17]. However, detrimental effects of these nonspecific, broad-spectrum protein synthesis and/or kinase inhibitors have been demonstrated. For instance, the developmental competence of bovine oocytes is compromised after blocking maturation at the germinal vesicle stage for 24 h with CHX or DMAP [19]. In contrast, purine derivatives (roscovitine, olomoucine), which specifically inhibit MPF and MAP kinase, have been used to

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delay meiotic progression of bovine oocytes without affecting their subsequent developmental potential to the blastocyst stage [20], suggesting their potential use for parthenogenetic activation.

Despite success with NT in the production of rhesus macaque infants [21], relatively little information is available concerning the efficacy of activation treatments on monkey MII oocytes and reconstructed embryos. Here we report efforts 1) to determine the efficiency of sequential calcium treatments (electric pulses or ionomycin) combined with protein synthesis or phosphorylation inhibitors (CHX or DMAP) in inducing parthenogenetic activation and development of rhesus macaque parthenotes; 2) to investigate the effect of ionomycin followed by roscovitine, a specific MPF inhibitor on parthenogenetic activation of monkey oocytes; and 3) to evaluate the effectiveness of these activation treatments in reconstructed embryos following NT.

MATERIALS AND METHODS

Animals

Mature rhesus macaque males and females housed in individual cages were used in this study. All animal procedures were approved by the Institutional Animal Care and Use Committee at the Oregon Regional Primate Research Center/Oregon Health Sciences University.

Follicular Stimulation and Oocyte Collection

One to 4 days following the onset of menses, adult rhesus macaque females were subjected to a follicular stimulation protocol [22, 23]. Briefly, monkeys received twice-daily injections of recombinant human FSH (rhFSH; 30 IU i.m.) and once-daily injections of Antide (a GnRH antagonist; 0.5 mg/kg s.c.) for 9 consecutive days. On the last 2 days of rhFSH/Antide stimulation, animals also received twice-daily injections of recombinant human LH (rhLH; 30 IU i.m.). On the last or next-to-last day of hormonal stimulation, ovarian morphology was recorded by ultrasonography (Advanced Technology Laboratories, ATL; Bothell, WA). Monkeys responding to follicular stimulation (follicles ≥4 mm diameter) received an injection of recombinant hCG (rhCG; 1000 IU i.m.) to induce oocyte maturation. Cumulus-oocyte complexes were collected from anesthetized animals by laparoscopic follicular aspiration (32–33 h post-rhCG) and placed in Hepes-buffered TALP (modified Tyrode solution with albumin, lactate, and pyruvate) medium [24] containing 0.3% BSA (TH3) at 37°C. Unless indicated otherwise, all hormones were from Ares Advanced Technologies Inc. (Norwell, MA) and other reagents were from Sigma-Aldrich Co. (St. Louis, MO). Oocytes, stripped of cumulus cells by mechanical pipetting after brief exposure (<1 min) to hyaluronidase (0.5 mg/ml), were placed in CMRL (Connaught Medical Research Laboratories; Life Technologies, Rockville, MD) medium at 37°C in 5% CO_2 balance air containing 10% fetal bovine serum (FBS; HyClone, Logan, UT), 10 mM L-glutamine (Life Technologies), 5 mM sodium pyruvate, 1 mM sodium lactate, 100 units/ml of penicillin (Life Technologies) and 100 μg/ml of streptomycin (Life Technologies) until further use [25].

Intracytoplasmic Sperm Injection

A cohort of MII oocytes from each experiment was fertilized by intracytoplasmic sperm injection (ICSI) [22] and used as controls to evaluate second polar body extrusion, pronuclear formation, cleavage, and development to the blastocyst stage. In contrast to conventional in vitro fertilization (IVF), ICSI allowed the use of cumulus-free MII oocytes and better visualization of second polar body extrusion and pronuclear formation. ICSI was performed within 35–36 h post-hCG injection employing sperm collected by penile electrostimulation from pregnancy-proven males [26]. Sperm were processed by centrifugation (200 × *g* for 7 min) of the liquid portion of the ejaculate, and the sperm pellet was resuspended in TH3. Before final resuspension, an aliquot was taken, and sperm motility and concentration was determined. Sperm concentrations were adjusted to 5 million motile sperm per milliliter in TH3 and stored, on average, 3 h at room temperature, prior to ICSI.

A mineral oil-covered, micromanipulation chamber containing a 30-μl TH3 drop with oocytes and a 4-μl drop of 10% polyvinylpyrrolidone (Irvine Scientific, Santa Ana, CA) diluted with 1 μl sperm was placed on the stage of an inverted microscope (Olympus IX70; Olympus America Inc., Melville, NY) equipped with micromanipulators (Narishige, Japan). An individual sperm was immobilized, aspirated into an ICSI pipette (Humagen, Charlottesville, VA), and injected into the oocyte cytoplasm, away from the polar body.

Parthenogenetic Activation

Cumulus-free MII oocytes, 35–36 h post-hCG injection, were randomly assigned to one of the following activation treatments: 1) exposure to 5 μM ionomycin (Calbiochem, La Jolla, CA) for 2 min (unless indicated otherwise) in TALP/Hepes medium supplemented with 1 mg/ml BSA and then washed for 5 min in TALP/Hepes supplemented with 30 mg/ml BSA (ionomycin alone); some ionomycin-treated oocytes were then transferred into CMRL medium containing 2) 2 mM DMAP (ionomycin/DMAP) or 3) 50 μM roscovitine (Calbiochem; ionomycin/roscovitine) and cultured at 37°C in 5% CO_2 balance air for 4 h. Alternatively, 4) some oocytes were electroporated three times, 30 min apart with two 50-μsec direct current pulses of 2.7 kV/cm (Electro Square Porator T-820; BTX, Inc., San Diego, CA) in 0.25 M D-sorbitol buffer containing 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 0.5 mM Hepes, and 1 mg/ml fatty acid-free BSA with incubation in CMRL medium containing 7.5 μg/ml CHX and 7.5 μg/ml CytoB between electroporations (electroporation/CHX/CytoB).

Nuclear Transfer Procedures

Blastomeres of Day 3 (Day 0 = day of fertilization) ICSI-produced rhesus monkey embryos were used as the source of donor nuclei. Zonae pellucidae were removed by brief exposure to 0.5% pronase and blastomeres were mechanically disaggregated after incubation in 0.5 mM EDTA in Ca^{2+}-free and Mg^{2+}-free Dulbecco PBS (Life Technologies) for 5–10 min. Recipient MII oocytes were incubated for 5 min with 5 μg/ml Hoechst 33342, transferred to 30 μl of TH3 containing 3.5 μg/ml cytochalasin D, and incubated for 10–15 min before enucleation. The first polar body and metaphase spindle were drawn into an enucleation pipette (25–28 μm outer diameter) with subsequent confirmation of removal of the Hoechst-stained metaphase spindle by epifluorescent microscopy. The time of exposure to UV light was restricted to less than 10 sec. A blastomere transfer pipette (33–35 μm outer diameter) was used to aspirate a disaggregated donor blastomere and transfer it into the perivitelline space of the cytoplast. Fusion of NT pairs was induced by two 50-μsec direct current pulses of 2.7 kV/cm (Electro Square Porator T-820) in D-sorbitol buffer. Fusion was evaluated visually 45–60 min after electroporation by noting the presence or absence of donor blastomeres in the perivitelline space. Fused NT embryos were activated approximately 2 h after fusion by exposure to either ionomycin/DMAP or ionomycin/roscovitine as indicated above and cultured in CMRL medium.

Embryo Culture

ICSI-produced embryos, parthenotes, and NT embryos (36–37, 39–40, and 42–43 h post-hCG injection, respectively) were placed in 4-well dishes (Nalge Nunc International Co., Naperville, IL) and cocultured at 37°C in 5% CO_2 balance air on buffalo rat liver (BRL) cell monolayers (25 000 cells per well) in CMRL medium. Extrusion of a second polar body and pronuclear formation were monitored and recorded every 2 h during 12-h period postactivation (hpa) or post-ICSI, and the progression of embryo growth was recorded daily. Embryos were transferred to fresh plates of BRL cells every other day for a maximum of 8 days. Development based on cleavage, morula, and blastocyst rates was assessed for each replicate and expressed relative to the number of pronuclear stage embryos or activated oocytes (second polar body appearance in ionomycin-alone group).

Statistical Analysis

Results, expressed as means and standard errors of the mean, were analyzed using one-way ANOVA and Fisher protected least significant difference test within Statview software (SAS Institute Inc., Cary, NC).

RESULTS

Meiotic Resumption and Pronuclear Formation Following Activation

Initially, the ionomycin exposure time necessary to induce parthenogenetic activation was defined. MII oocytes

TABLE 1. Activation protocols, meiotic resumption, and pronuclear formation in rhesus monkey MII oocytes.

Treatment	No. of oocytes[a]	Second polar body extrusion (mean % ± SEM)	Pronuclei (1 or more) (mean % ± SEM)
ICSI control	108	74 ± 4[b]	71 ± 5[b]
Ionomycin alone	10	70 ± 10[b]	0[c]
Ionomycin/DMAP	51	0[c]	82 ± 9[bd]
Electroporation/CHX/CytoB	18	0[c]	80 ± 20[bd]
Ionomycin/roscovitine	64	92 ± 6[d]	93 ± 6[d]

[a] The total number of oocytes in each treatment was derived from at least two replications.
[b,c,d] Treatments with different superscripts within a column are significantly different ($P < 0.05$).

at 35–36 h post-hCG injection were exposed to 5 μM ionomycin for 2, 3, or 4 min followed by incubation in 2 mM DMAP for 4 h. Similar levels of pronuclear formation, initial cleavage, and blastocyst formation were observed, however, up to 10% of oocytes lysed following the 4-min exposure (results not shown). Thus, a 2-min treatment with ionomycin was considered adequate, and this exposure time was routinely used in subsequent experimentation.

The efficacy of several parthenogenetic activation protocols was evaluated and compared to ICSI-fertilized "control" oocytes. Control oocytes completed meiosis and extruded a second polar body by 4 h (74% ± 4%) with the resultant zygotes forming two pronuclei at approximately 10 h after fertilization (71% ± 5%; Table 1; Fig. 1A). Treatment of oocytes with ionomycin alone induced the resumption of meiosis and second polar body extrusion (at 90 min) at a rate comparable to the control (70% ± 10%). However, oocytes in this group failed to form pronuclei. DMAP, CHX, or roscovitine exposure alone did not result in oocyte activation because neither second polar body nor pronuclear formation was observed (results not shown). Sequential treatments with ionomycin and DMAP or electroporation and CHX/cytoB induced pronuclear formation similar to the ICSI group; however, these oocytes did not complete meiosis or extrude a second polar body. Most of the parthenotes (>90%) in these two groups had a single pronucleus and one (first) polar body by 10 hpa (Fig. 1B). The remaining activated parthenotes (<10%) demonstrated a range of nuclear configurations with two, three, and more smaller pronuclei and one polar body (Fig. 1C). In contrast, the majority of oocytes treated sequentially with ionomycin followed by roscovitine extruded the second polar body and formed a single pronucleus. The percentage of activated oocytes containing a second polar body (92% ± 6%) or pronucleus (93% ± 6%) in this group was significantly higher than observed in ICSI controls (Table 1). The presence of chromatin in both polar bodies was confirmed by selective staining with a DNA-specific chromaphore (Hoechst) and epifluorescence microscopy (Fig. 1D).

In Vitro Development of Parthenotes

The developmental potential of activated oocytes containing either pronuclei or second polar bodies or both from each treatment as well as control zygotes was assessed (Table 2). Most pronuclear-stage parthenotes cleaved on Day 1—95%, 88%, and 61% for oocytes activated by ionomycin/DMAP, electroporation/CHX/CytoB, or ionomycin/roscovitine, respectively. These rates were comparable ($P > 0.05$) with those seen with ICSI controls (87%). Oocytes activated by ionomycin alone failed to cleave. Further development to the compact morula stage by Days 5–6 was at the control rate for all remaining groups. However, io-

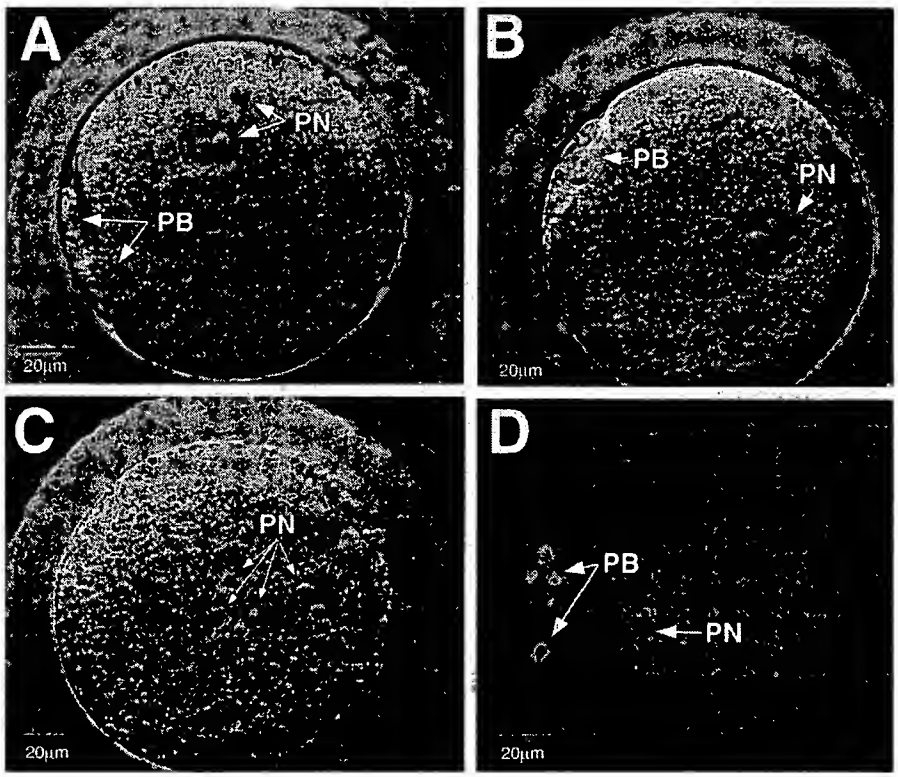

FIG. 1. A) Rhesus monkey zygote fertilized by ICSI with two pronuclei (PN) and two polar bodies (PB). B) Ionomycin/DMAP-activated parthenote with one PN and one PB. C) Ionomycin/DMAP-activated parthenote with multiple micro-PN. Hoffman optics. D) Epifluorescence microscopy of ionomycin/roscovitine-activated parthenote stained with Hoechst. Note the presence of a single PN and two PBs.

TABLE 2. In vitro development of rhesus monkey parthenotes.

Treatment	No. of oocytes	Cleavage (mean % ± SEM)	Compact moralu (mean % ± SEM)	Blastocyst (mean % ± SEM)
ICSI control	58	87 ± 5[b]	67 ± 6[bc]	54 ± 6[b]
Ionomycin alone[a]	7	0	0	0
Ionomycin/DMAP	35	95 ± 19[b]	78 ± 10[bd]	58 ± 14[b]
Electroporation/CHX/CytoB	14	88 ± 21[b]	73 ± 10[bc]	48 ± 15[b]
Ionomycin/roscovitine	29	61 ± 9[b]	41 ± 14[c]	25 ± 15[b]

[a] Activated oocytes are defined as oocytes with pronuclei or with a second polar body (ionomycin-alone group). The total number of activated oocytes in each treatment was derived from at least two replications.
[b,c,d] Treatments with different superscripts within a column were significantly different ($P < 0.05$).
[c] Treatment was excluded from statistical analysis.

nomycin/roscovitine treatment produced parthenotes that were significantly impaired when contrasted with ionomycin/DMAP-treated parthenotes (Table 2).

The proportion of parthenotes that reached the blastocyst stage by Days 7–8 of culture in the ionomycin/DMAP, electroporation/CHX/CytoB, and ionomycin/roscovitine groups did not differ ($P > 0.05$) from control ICSI-produced embryos (Table 2). Some parthenote blastocysts were indistinguishable from control embryos (Fig. 2, A and B), however, a high proportion of parthenotes were characterized as low quality based on their inability to reach expanded and hatched blastocyst stages, a small inner cell mass, the presence of extruded blastomeres in the blastocoelic cavity, and a high proportion of presumably apoptotic cells.

Activation of Reconstructed Embryos

The ability of ionomycin/DMAP or ionomycin/roscovitine to activate fused pairs was measured after NT of embryonic blastomeres. Two replicates of each treatment were performed and an enucleation efficiency of 100% was confirmed by epifluorescence microscopy. Within 40–60 min of the fusion pulse, 70% ± 4% (30/42) of NT pairs were fused. Two hours later, reconstructed embryos were assigned to one of two different activation treatments, ionomycin/DMAP or ionomycin/roscovitine. With ionomycin/DMAP, an 87% ± 18% pronuclear-formation rate was observed by 10 hpa, with the majority of activated NT embryos (58% ± 12%) possessing a single, large pronucleus (Fig. 3A). The remaining embryos contained two, three, or more small pronuclei and there was no polar body formation observed among activated NT embryos in this treatment. Activation with ionomycin/roscovitine yielded a pronuclear formation rate of 83% ± 24%, and all activated embryos contained one large pronucleus. However, 36% ± 19% of these embryos extruded one or two polar bodies (Fig. 3, B and C). The presence of DNA in these polar bodies was confirmed by Hoechst staining (Fig. 3D).

DISCUSSION

As shown here with rhesus monkey oocytes and by others with bovine oocytes [14, 16, 27, 28], the combination treatments of ionomycin/DMAP or electroporation/CHX/CytoB that first initiate a calcium flux and then inhibit either protein phosphorylation or protein synthesis, induce pronuclear formation without completion of meiosis. Instead, activated oocytes enter interphase of the first mitotic cycle in a pseudo-diploid state because there is no loss of chromosomes in a second polar body. Cycloheximide, when combined with the cytokinesis blocker, cytochalasin B, inhibited second polar body formation in bovine parthenotes with the formation of diploid oocytes containing two pronuclei [29] and led here to the recovery of rhesus monkey parthenotes with a single, presumably diploid pronucleus. The ability of monkey oocytes activated by ionomycin/DMAP or electroporation/CHX/cytoB to cleave and develop to compact morula and blastocyst stages at rates comparable with sperm-fertilized, ICSI controls suggests that embryonic developmental competence is not compromised by such exposures.

Exposure of monkey oocytes to ionomycin alone induced activation with resumption and completion of meiosis without pronuclear formation. This could reflect reentry of activated oocytes into a new M-phase arrest known as metaphase III [14, 30]. In this case, artificially induced increases in intracellular calcium cause rapid but transient inactivation of MPF. A decline in MPF in turn allows activated oocytes to resume meiosis and expel a second polar body; however, subsequent protein synthesis and phos-

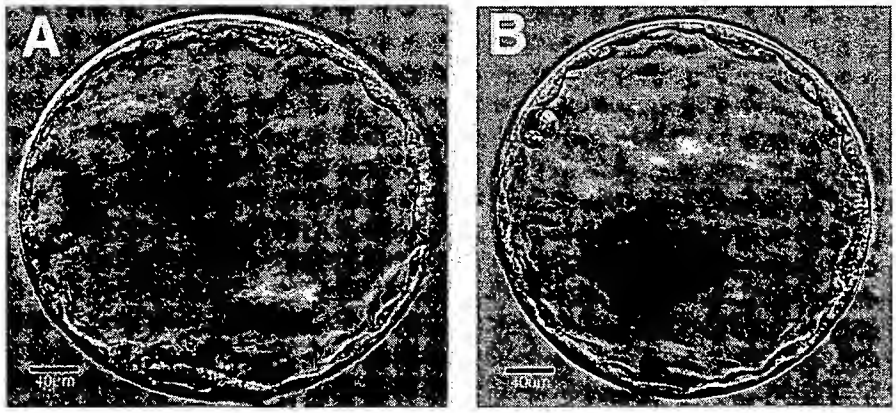

FIG. 2. A) Expanded blastocyst produced by ICSI, Day 8 of development. B) Parthenote at the expanded blastocyst stage produced by ionomycin/DMAP activation, Day 8 of development. Hoffman optics.

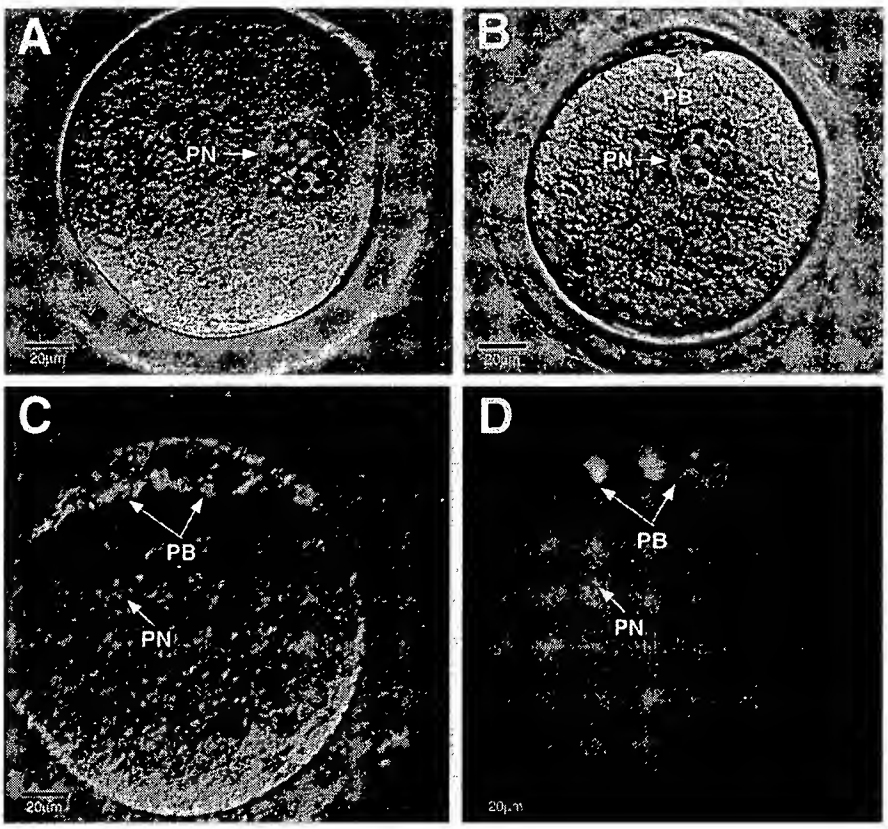

FIG. 3. A) Reconstructed NT embryo at the pronuclear stage following activation by ionomycin/DMAP. Note the presence of a large, prominent pronucleus (PN) containing multiple nucleoli. B) Reconstructed NT embryo activated by ionomycin/roscovitine. Note the presence of one PN and a single polar body (PB). C) ionomycin/roscovitine-activated NT embryo containing a single PN and two PBs. Hoffman optics. D) Epifluorescence microscopy of the NT embryo stained with Hoechst and confirming the presence of DNA in the PN and both PBs.

phorylation restores MPF activity causing recondensation of chromosomes and metaphase III arrest [18, 29]. Conversely, elimination of the ionomycin exposure step (i.e., DMAP or cycloheximide alone), did not support oocyte activation, consistent with previous experience with bovine oocytes [14, 16, 27, 29].

Studies were also conducted with roscovitine, a purine derivative, which selectively inhibits MPF by preventing ATP binding to the $p34^{cdc2}$ subunit of MPF without affecting the activity of several kinases, including protein kinase A, G, and C isoforms; myosin light-chain kinase; casein kinase 2; insulin receptor tyrosine kinase; c-src; v-abl; and the MAP kinases erk1 and erk2 [31]. Treatment with roscovitine, after ionomycin activation, allowed monkey MII oocytes to resume and complete meiosis. However, oocytes so treated both extruded a second polar body and formed a single pronucleus (Table 1), suggesting that roscovitine exposure did not interfere with reorganization of cytoskeletal elements during completion of the second meiotic division. To our knowledge, this is the first report that a sequential treatment with ionomycin followed by roscovitine induces parthenogenetic activation and formation of pronuclei following chromosome segregation. Although high activation and initial cleavage rates were observed after ionomycin/roscovitine treatment, parthenotes in this group displayed low developmental potential to the compact morula stage compared to ionomycin/DMAP treatment. This outcome may reflect the haploid nature of embryos produced by this treatment [13, 32].

Based on the nuclear configurations observed after oocyte activation with ionomycin/DMAP or ionomycin/roscovitine, we hypothesized that similar results would be obtained with NT embryos. Indeed, polar body extrusion was not observed after activation of monkey NT embryos with ionomycin/DMAP, and most of the activated embryos in this group displayed a single pronucleus. NT embryos activated with ionomycin/roscovitine also contained exclusively one, large pronucleus, however, and in contrast, a third of these embryos abstricted one or two polar bodies containing chromatin material, as detected by Hoechst staining. The ploidy of these embryos has not yet been determined.

Extrusion of a polar body after activation of NT embryos has been observed in mice [33–35]. It is interesting that emission of the polar body depended on the cell cycle stage of the donor blastomeres. Most of the mouse NT embryos that received embryonic nuclei in an early cell cycle stage (corresponding to G1 phase) formed a single diploid pronucleus with no polar body. In contrast, late stage donor nuclei (presumably G2 phase) primarily formed a single diploid pronucleus and one diploid polar body [35]. Separation of the polar body could reflect the continuation of mitotic events in the donor nucleus serving as a mechanism of autoregulation of ploidy in the resulting NT embryos [34, 35]. Advantage was taken of this unusual phenomenon to produce viable mouse clones from G2- or M-phase donor nuclei [36, 37]. The cell cycle stage of donor blastomeres used in the present study is unknown because unsynchronized 8- to 16-cell stage embryos were utilized. However, based on the cited observations of mouse NT embryos, it is likely that monkey NT embryos that emitted a second polar body originated from late S-, G2-, or M-phase donor nuclei, whereas the remaining embryos that did not expel a polar body received G1 phase blastomeres.

In summary, current procedures for cloning mammals by nuclear transfer are predicated on the use of cells ar-

rested in the G0/G1 phase of the cell cycle [1, 2, 4, 5, 38], although G2 or nuclei at the metaphase stage have been used in the mouse [36, 37, 39]. Due to the lack of efficient activation mechanisms allowing chromosome segregation into pseudo polar bodies and restoration of the 2N genomic complement, the use of late S-, G2-, or metaphase-stage donor nuclei for NT has not been possible in cattle, sheep, and monkey [1–4, 21]. The approach tested here with ionomycin/roscovitine offers a unique tool for studying the developmental events of NT embryos receiving donor nuclei at different cell cycle stages and could be particularly valuable for improving somatic cell cloning efficiency. We identified simple yet effective methods of oocyte activation in the monkey: ionomycin/DMAP, electroporation/CHX/CytoB, and ionomycin/roscovitine, which are suitable for cytoplasts or reconstructed embryos. Ionomycin/roscovitine activation could also be useful in the bovine, where injection of spermatozoa must be accompanied by artificial oocyte activation in order to achieve normal fertilization events [40] or in the monkey, in which frozen-thawed sperm show reduced fertilizing ability following ICSI [41].

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ological grouping of the genotypes throughout the epidemic (Fig. 2) (table S2).

In tracing the molecular evolution of SARS-CoV in China, we observed that the epidemic started and ended with deletion events, together with a progressive slowing of the nonsynonymous mutation rates and a common genotype that predominated during the latter part of the epidemic. The mechanistic explanation for the selective adaptation and purification processes that led to such genomic evolutionary changes in SARS-CoV requires further work (*29*). Nonetheless, this study has provided valuable clues to aid further investigation of this remarkable evolutionary tale.

We have sequenced the complete S gene (GenBank accession number AY525636) from an oropharyngeal swab sample (sampling date, 22 December 2003) collected from the most recent index patient of the city of Guangzhou (onset date, 16 December 2003; hospitalized 20 December 2003; www.wpro.who.int/sars/docs/pressreleases/pr_27122003.asp). Phylogenetic analysis of this S gene sequence with those from the human SARS-CoV and palm civet SARS-like coronavirus indicated that this most recent case of SARS-CoV is much closer to the palm civet SARS-like coronavirus than to any human SARS-CoV detected in the previous epidemic (fig. S7 and table S4). Because it is evidently different from the recent laboratory infections in Singapore (www.who.int/csr/don/2003_09_24/en) and Taiwan (www.who.int/mediacentre/releases/2003/np26/en), it strengthens the argument for animal origin of the human SARS epidemic.

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20. The SARS-CoV sequence with the 415-nt deletion (CUHK-LC2, CUHK-LC3, CUHK-LC4, and CUHK-LC5) was obtained from two SARS patients whose disease was linked to e late cluster of SARS cases in Hong Kong. Both patients had disease onset in mid-May 2003. The CUHK-LC2 sequence was initially obtained from the culture isolate of a throat wash specimen of an infected hospital health care worker and was later confirmed from the same specimen directly. CUHK-LC3, CUHK-LC4, and CUHK-LC5 were obtained from three different nasal swab specimens both directly and from the culture supernatants of an elderly patient who acquired SARS in the same hospital.
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Evidence of a Pluripotent Human Embryonic Stem Cell Line Derived from a Cloned Blastocyst

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Somatic cell nuclear transfer (SCNT) technology has recently been used to generate animals with a common genetic composition. In this study, we report the derivation of a pluripotent embryonic stem (ES) cell line (SCNT-hES-1) from a cloned human blastocyst. The SCNT-hES-1 cells displayed typical ES cell morphology and cell surface markers and were capable of differentiating into embryoid bodies in vitro and of forming teratomas in vivo containing cell derivatives from all three embryonic germ layers in severe combined immunodeficient mice. After continuous proliferation for more than 70 passages, SCNT-hES-1 cells maintained normal karyotypes and were genetically identical to the somatic nuclear donor cells. Although we cannot completely exclude the possibility that the cells had a parthenogenetic origin, imprinting analyses support a SCNT origin of the derived human ES cells.

The isolation of pluripotent human embryonic stem (ES) cells (*1*) and breakthroughs in somatic cell nuclear transfer (SCNT) in mammals (*2*) have raised the possibility of performing human SCNT to generate potentially unlimited sources of undifferentiated cells for use in research, with potential applications in tissue repair and transplantation medicine. This concept, known as "therapeutic cloning," refers to the transfer of the nucleus of a somatic cell into an enucleated donor oocyte (*3*). In theory, the oocyte's cytoplasm would reprogram the transferred nucleus by silencing all the somatic cell genes and activating the embryonic ones. ES cells would be isolated from the inner cell mass (ICM) of the cloned preimplantation embryo. When applied in a therapeutic setting, these cells would carry the nuclear genome of the patient; therefore, it is proposed that after directed cell differentiation, the cells could be transplanted without immune rejection to treat degenerative disorders such as diabetes, osteoarthritis, and Parkinson's disease

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(among others). Previous reports have described the generation of bovine ES-like cells (*4*) and mouse ES cells from the ICMs of cloned blastocysts (*5*–*7*) and the development of cloned human embryos to the 8- to 10-cell stage (*8*, *9*). Here we describe evidence of the derivation of human ES cells after SCNT (*10*).

Fresh oocytes and cumulus cells were donated by healthy women for the express purpose of SCNT stem cell derivation for therapeutic cloning research and its applications. Before beginning any experiments, we obtained approval for this study from the Institutional Review Board on Human Subjects Research and Ethics Committees (Hanyang University Hospital, Seoul, Korea). Donors were fully aware of the scope of our study and signed an informed consent form (a summary of the informed consent form is available in the supporting online text); donors voluntarily donated oocytes and cumulus cells (including DNA) for therapeutic cloning research and its applications only, not for reproductive cloning; and there was no financial payment. A total of 242 oocytes were obtained from 16 volunteers (there were one or two donors for each trial) after ovarian stimulation: 176 metaphase II (MII) oocytes were used directly for SCNT, whereas the remaining 66 oocytes were allowed to mature to the MII stage before use in SCNT. Autologous SCNT was performed; that is, the donor's own cumulus cell, isolated from the cumulus-oocyte complex (COC), was transferred back into the donor's own enucleated oocyte. Before enucleation, the oocytes were matured in vitro in G1.2 medium (Vitro Life, Goteborg, Sweden) for 1 to 2 hours. Enucleation, SCNT, and electrical fusion were performed as described (*11*). To directly confirm that the oocyte's DNA was removed during enucleation, we imaged the extruded DNA MII spindle complex from every oocyte with Hoechst 33342 fluorescent DNA dye (Fig. 1, A and B; arrows).

Without any report of an efficient protocol for human SCNT, several critical steps had to be optimized (*2*), including reprogramming time, activation method, and in vitro culture conditions. Reprogramming time, or the lapse of time between cell fusion and egg activation, returns the gene expression of the somatic cell to that needed for appropriate embryonic development. Initially, we investigated the effect of simultaneous fusion and activation, as used for porcine SCNT (*12*, *13*), but observed low fusion and cleavage rates, with no blastocyst development. Instead, we adapted the bovine SCNT procedure of waiting a few hours between fusion and activation. By allowing 2 hours for reprogramming, we were able to develop ~25% of the embryos to the blastocyst stage.

Because sperm-mediated activation is absent in SCNT, an artificial stimulus is needed to initiate development. Various chemical, physical, and mechanical agents induce parthenogenetic development in mice (*14*), but human data are limited. Oocyte activation using the calcium ionophore A23187 (calcimycin) or ionomycin and the protein synthesis inhibitor puromycin induces parthenogenetic development of human oocytes at different efficiencies (*15*). We found that incubation in 10 μM A23187 for 5 min, followed by incubation with 2.0 mM 6-dimethylaminopurine (DMAP) for 4 hours, gave efficient chemical activation for human SCNT eggs. Other investigators have reported encouraging results in overcoming inefficiencies in embryo culture by supplementing the culture with different energy substrates (*16*). Furthermore, the recent development of serum-free sequential media has led to considerable improvement in the rate of clinical pregnancies produced by in vitro fertilization (IVF) (*17*). In this study, human modified synthetic oviductal fluid with amino acids (hmSOFaa) was prepared by supplementing mSOFaa (*18*) with human serum albumin (10 mg/ml) and fructose (1.5 mM) instead of bovine serum albumin (8 mg/ml) and glucose (1.5 mM). The replacement of glucose with fructose improves the developmental competence of bovine SCNT embryos (*11*, *19*). Culture of human SCNT-derived embryos in G1.2 medium for the first 48 hours followed by hmSOFaa medium produced more blastocysts, as compared to culture in G1.2 medium for the first 48 hours followed by culture in G1.2 medium or in continuous hmSOFaa medium (Table 1). Cibelli *et al.* (*8*) reported that the treatment of human oocytes with 5 μM calcium ionomycin followed by 2 mM DMAP in G1.2 culture medium triggered pronucleus formation, embryonic cleavage, and the formation of a blastocoelic cavity in human parthenotes. However, they did not obtain human SCNT blastocysts when their protocol was applied to SCNT embryos. Limitations in oocyte supply precluded full optimization of all the parameters for human SCNT; nonetheless, the protocol described here produced cloned blastocysts at rates of 19 to 29% (as a percentage of oocytes used) and was comparable to those produced by established SCNT methods in cattle (~25%) (*11*) and pigs (~26%) (*12*, *13*).

A total of 30 SCNT-derived blastocysts were cultured, 20 ICMs were isolated by immunosurgical removal of the trophoblast, and one ES cell line (SCNT-hES-1) was derived. The resulting SCNT-hES-1 cells had a high nucleus-to-cytoplasm ratio and prominent nucleoli. The cell colonies displayed similar morphology to that reported previously for hES cells derived after IVF (Fig. 1, C to E). When cultured in defined medium conditioned for neural cell differentiation (*20*), SCNT-hES-1 cells differentiated into nestin-positive cells, an indication of primitive neuroectoderm differentiation (Fig. 1F). The SCNT-hES-1 cell line was mechanically passaged by dissociation every 5 to 7 days and successfully maintained its undifferentiated mor-

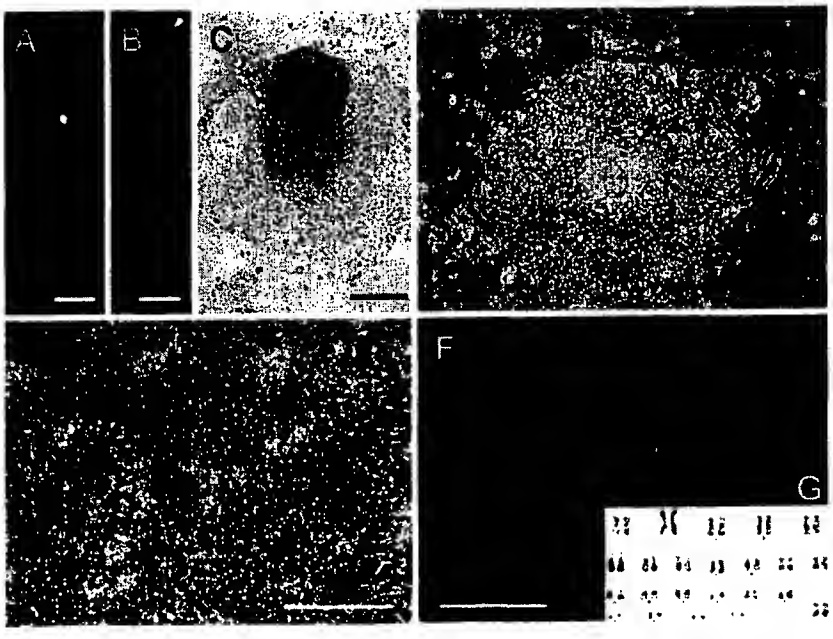

Fig. 1. Confirmation of enucleation, photographs of human SCNT ES cells and their differentiated progeny, and karyotype analysis. (A and B) Images (×200) of extruded DNA MII spindle complexes (arrows) from an oocyte before (A) and after (B) enucleation. (C to E) Bright-field [(C), ×100] and phase contrast [(D), ×100] micrographs and higher magnification image [(E), ×200] of a colony of SCNT-hES-1 cells. Immunofluorescence staining for nestin [(F), ×200] and G-banded kayotyping (G) in SCNT-hES-1 cells are shown. Scale bars, 20 μm in (A) and (B) and 100 μm in (C) to (F).

phology after continuous proliferation for >70 passages, while still maintaining a normal female (XX) karyotype (Fig. 1G) (*21*). Furthermore, the SCNT-hES-1 cells expressed ES cell markers such as alkaline phosphatase, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and Oct-4, but not SSEA-1 (Fig. 2). As previously described in monkey (*22*) and human ES cells (*1*, *23*, *24*) and in mouse SCNT-ES cells (*6*), SCNT-hES-1 cells did not respond to exogenous leukaemia inhibitory factor, suggesting that a pluripotent state is maintained by a gp130-independent pathway. The pluripotency of SCNT-hES-1 cells was investigated in vitro (fig. S1) and in vivo (Fig. 3). Clumps of the cells were cultured in vitro in suspension to form embryoid bodies. The resulting embryoid bodies were stained with three dermal markers and were found to differentiate into a variety of cell types, including derivatives of endoderm, mesoderm, and ectoderm (fig. S1). When undifferentiated SCNT-hES-1 cells were injected into the testes of severe combined immunodeficient (SCID) mice, teratomas were obtained 6 to 7 weeks after injection. The resulting teratomas contained tissue representative of all three germ layers. Differentiated tissues seen in Fig. 3 include neuroepithelial rosset, pigmented retinal epithelium, smooth muscle, bone, cartilage, connective tissues, and glandular epithelium. The DNA fingerprinting analysis with human short tandem repeat (STR) markers indicates that the cell line originated from the cloned blastocysts reconstructed from the donor cells, not from parthenogenetic activation (Fig. 4, A to C). The statistical probability that the cells may have derived from an unrelated donor is 8.8×10^{-16}. Reverse transcription polymerase chain reaction (RT-PCR) amplification for paternally expressed (*hSNRPN* and *ARH1*) and maternally expressed (*UBE3A* and *H19*) genes further confirmed that the cell line originated from the donor cells (Fig. 4D).

Simerly *et al.* (*26*) recently reported defective mitotic spindles after SCNT in nonhuman primate embryos, perhaps resulting from the depletion of microtubule motor and centrosome proteins lost to the meiotic spindle after enucleation. In this study, Fig. 1G demonstrates that SCNT-hES-1 cells have the normal karyotype. We speculate that SCNT blastocysts from which ES cell lines were not derived might have been aneuploid. However, it is important to note that our investigations differ from those of Simerly *et al.* in a few ways: Media and protocols for in vitro development were optimized for human oocytes and embryos, whereas the protocols for nonhuman primate studies are extrapolated from clinical procedures; the enucleation method here differs, because we squeeze the MII oocyte so that the DNA spindle complex is extruded through a small hole in the zona pellucida, instead of aspirating the DNA spindle complex with a glass pipette as others have described (*27*); and the DNA spindle complex is extruded shortly after the appearance of the first polar body, so that it may even be at the prometaphase II stage.

In this report, we provide three lines of evidence supporting the nuclear transfer origins of the SCNT-hES-1 cell line: (i) DNA extraction was verified for each of the 242 enucleated oocytes (Fig. 1, A and B; arrows); (ii) DNA fingerprinting showed heterozy-

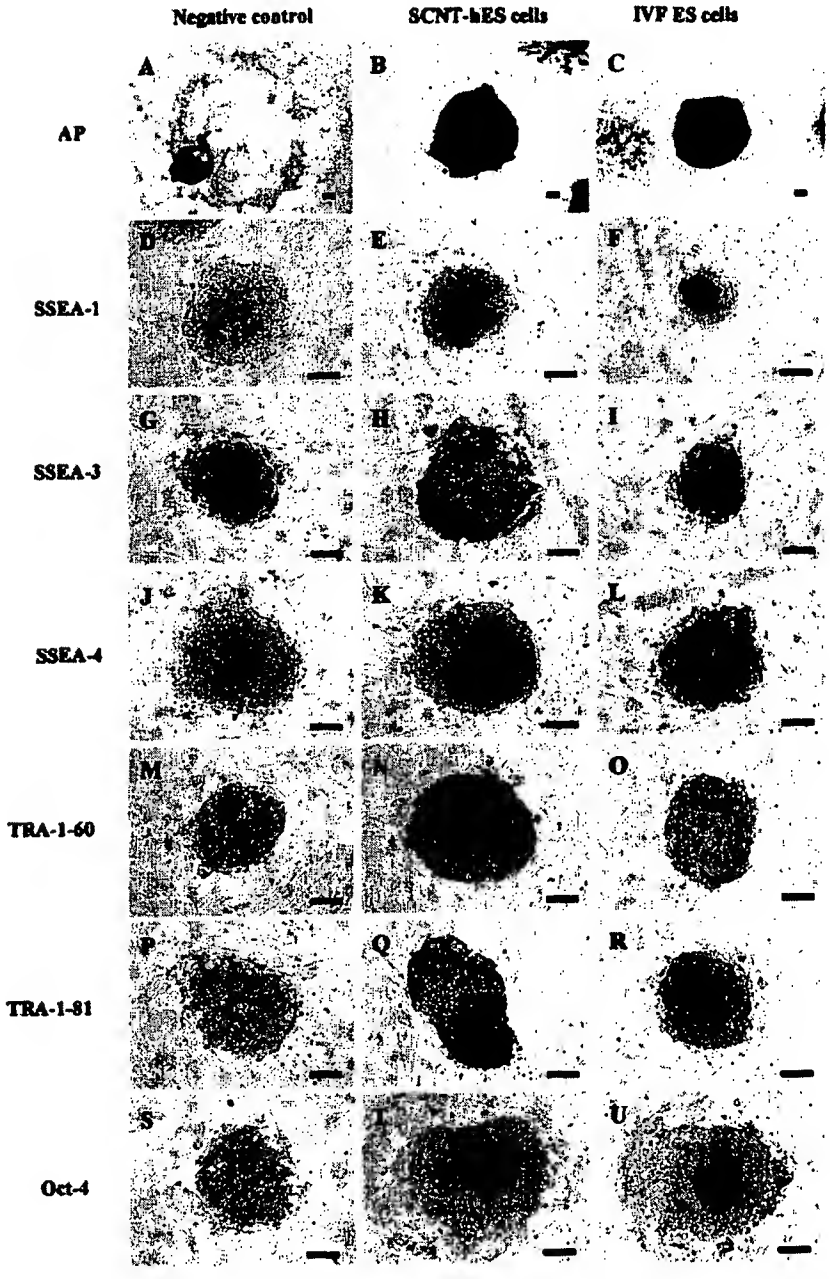

Fig. 2. Expression of characteristic cell surface markers in human SCNT ES cells. SCNT-hES-1 cells expressed cell surface markers, including alkaline phosphatase (B), SSEA-3 (H), SSEA-4 (K), TRA-1-60 (N), TRA-1-81 (Q), and Oct-4 (T), but not SSEA-1 (E). The differentiated SCNT-hES-1 cells were not stained with alkaline phosphatase (A). The IVF-derived human ES cells (Miz-hES) were used for comparison and also expressed alkaline phosphatase (C), SSEA-3 (I), SSEA-4 (L), TRA-1-60 (O), TRA-1-81 (R), and Oct-4 (U), but not SSEA-1 (F). Negative controls not treated with first antibodies are shown (D, G, J, M, P, and S). Magnification in (A) to (U), ×40. Scale bars, 100 μm.

gous, not homozygous, chromosomes (Fig. 4, A to C); and (iii) RT-PCR showed biparental, and not unimaternal, expression of imprinted genes (Fig. 4D). Although the Cyno-1 parthenogenetic cells retained their strictly maternal imprints, that evidence came from a single monkey cell line. Given the aberrant expression of imprinted genes after murine SCNT (*28*), perhaps the SCNT-hES-1 cells' biparental expression of imprinted genes might have been influenced by SCNT or subsequent culture. Heterologous along with autologous SCNT will provide more definitive molecular evidence. Although overwhelming ethical constraints preclude any reproductive cloning attempts, complementary investigations in nonhuman primates might provide additional and confirmatory information. Consequently, although we cannot exclude the possibility of a parthenogenetic origin, the studies reported here support the conclusion that the SCNT-hES-1 cell line originated from the donor's diploid somatic cumulus cell after SCNT.

In order to successfully derive immunocompatible human ES cells from a living donor, a reliable and efficient method for producing cloned embryos and ES isolation must be developed. Thomson *et al.* (*1*), Reubinoff *et al.* (*23*), and Lanzendorf *et al.* (*29*) produced human ES cell lines at high efficiency. Briefly, five ES cell lines were derived from a total of 14 ICMs, two ES cell lines were derived from four ICMs, and three ES cell lines were derived from 18 ICMS, respectively. In our study, one SCNT-hES cell line was derived from 20 ICMs. It remains to be determined whether this low efficiency is due to faulty reprogramming of the somatic cells or to subtle variations in our experimental procedures. We cannot rule out the possibility that the genetic background of the cell donor had an impact on the overall efficiency of the procedure. Further improvements in SCNT protocols and in vitro culture

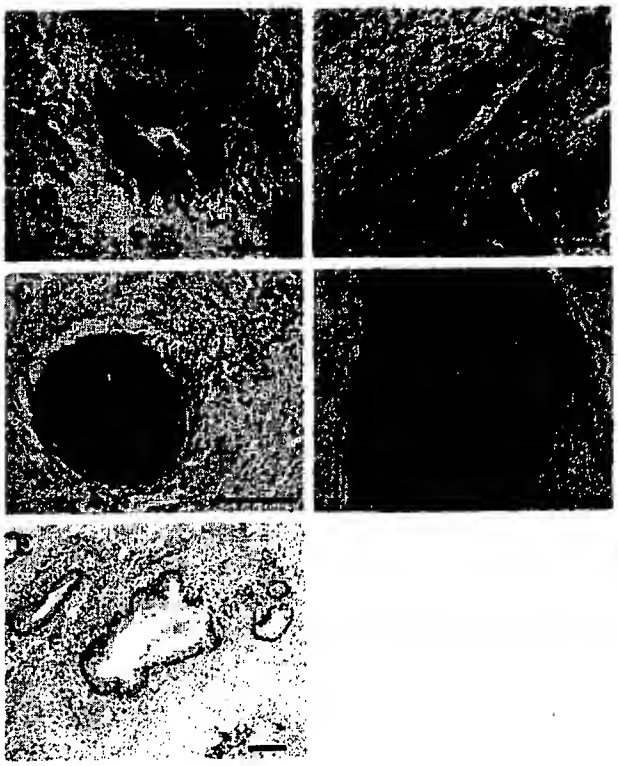

Fig. 3. Teratomas formed by human SCNT ES cells in the testes of SCID mice at 12 weeks after injection. Neuroepithelial rosset (A), pigmented retinal epithelium (B), ostoid island showing bony differentiation (C), cartilage (D), and glandular epithelium with smooth muscle and connective tissues (E). Magnification in (A) to (D), ×200; in (E), ×100. Scale bar, 100 μm.

Table 1. Conditions for human SCNT.

Experiment	Activation condition*		Reprogramming time (hours)	1st step medium†	2nd step medium	No. of oocytes	No. (%) of cloned embryos developed to		
							Two-cell	Compacted morula	Blastocyst
1st set	10 μM ionophore	6-DMAP	2	G 1.2	hmSOFaa	16	16 (100)	4 (25)	4 (25)
	10 μM ionophore	6-DMAP	4	G 1.2	hmSOFaa	16	15 (94)	1 (6)	0
	10 μM ionophore	6-DMAP	6	G 1.2	hmSOFaa	16	15 (94)	1 (6)	1 (6)
	10 μM ionophore	6-DMAP	20	G 1.2	hmSOFaa	16	9 (56)	1 (6)	0
2nd set	10 μM ionophore	6-DMAP	2	G 1.2	hmSOFaa	16	16 (100)	5 (31)	3 (19)
	5 μM ionophore	6-DMAP	2	G 1.2	hmSOFaa	16	11 (69)	0	0
	10 μM ionomycin	6-DMAP	2	G 1.2	hmSOFaa	16	12 (75)	0	0
	5 μM ionomycin	6-DMAP	2	G 1.2	hmSOFaa	16	9 (56)	0	0
3rd set	10 μM ionophore	6-DMAP	2	G 1.2	hmSOFaa	16	16 (100)	4 (25)	3 (19)
	10 μM ionophore	6-DMAP	2	G 1.2	G 2.2	16	16 (100)	0	0
	10 μM ionophore	6-DMAP	2	Continuous	hmSOFaa	16	16 (100)	0	0
4th set	10 μM ionophore	6-DMAP	2	G 1.2	hmSOFaa	66	62 (93)	24 (36)	19 (29)

*Fused donor oocytes and somatic cells were activated in either calcium ionophore A23187 (5 or 10 μM) or ionomycin (5 or 10 μM) for 5 min, followed by 2 mM 6-DMAP treatment for 4 hours. †Oocytes were incubated in the first medium for 48 hours.

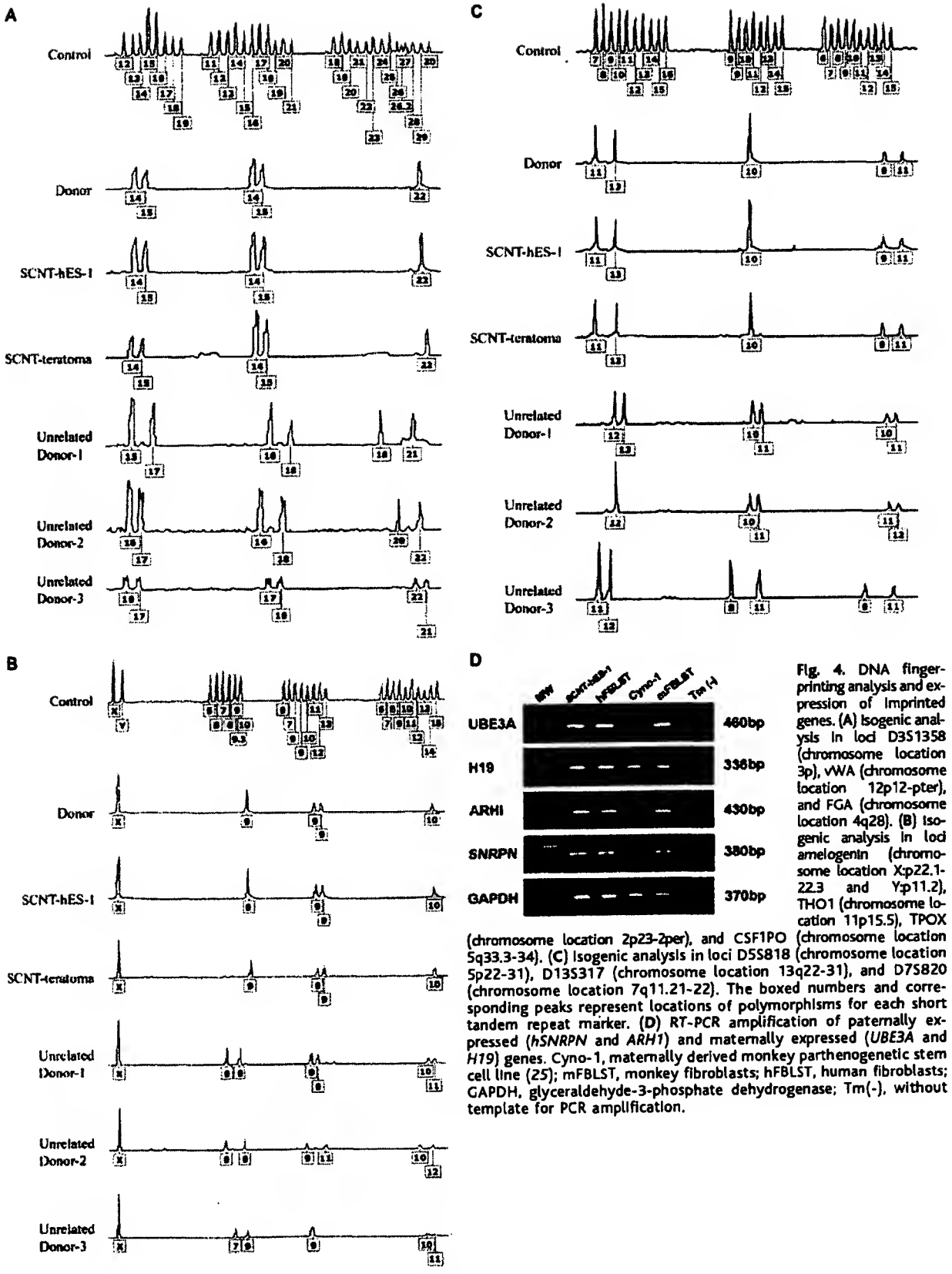

Fig. 4. DNA fingerprinting analysis and expression of imprinted genes. (A) Isogenic analysis in loci D3S1358 (chromosome location 3p), vWA (chromosome location 12p12-pter), and FGA (chromosome location 4q28). (B) Isogenic analysis in loci amelogenin (chromosome location X:p22.1-22.3 and Y:p11.2), THO1 (chromosome location 11p15.5), TPOX (chromosome location 2p23-2per), and CSF1PO (chromosome location 5q33.3-34). (C) Isogenic analysis in loci D5S818 (chromosome location 5p22-31), D13S317 (chromosome location 13q22-31), and D7S820 (chromosome location 7q11.21-22). The boxed numbers and corresponding peaks represent locations of polymorphisms for each short tandem repeat marker. (D) RT-PCR amplification of paternally expressed (*hSNRPN* and *ARH1*) and maternally expressed (*UBE3A* and *H19*) genes. Cyno-1, maternally derived monkey parthenogenetic stem cell line (*25*); mFBLST, monkey fibroblasts; hFBLST, human fibroblasts; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Tm(-), without template for PCR amplification.

systems are needed before contemplating the use of this technique for cell therapy. In addition, the mechanisms governing the differentiation of human tissues must be elucidated in order to produce tissue-specific cell populations from undifferentiated ES cells. This study shows the feasibility of generating human ES cells from a somatic cell isolated from a living person.

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Force-Clamp Spectroscopy Monitors the Folding Trajectory of a Single Protein

Julio M. Fernandez* and Hongbin Li

We used force-clamp atomic force microscopy to measure the end-to-end length of the small protein ubiquitin during its folding reaction at the single-molecule level. Ubiquitin was first unfolded and extended at a high force, then the stretching force was quenched and protein folding was observed. The folding trajectories were continuous and marked by several distinct stages. The time taken to fold was dependent on the contour length of the unfolded protein and the stretching force applied during folding. The folding collapse was marked by large fluctuations in the end-to-end length of the protein, but these fluctuations vanished upon the final folding contraction. These direct observations of the complete folding trajectory of a protein provide a benchmark to determine the physical basis of the folding reaction.

Resolving the folding pathway of a protein remains a challenge in biology (*1*–*9*). Here, we demonstrate a method by which the entire folding trajectory of a single protein can be recorded as a function of time. We used single-molecule atomic force microscopy techniques (*10*, *11*) in the force-clamp mode (*12*, *13*) to apply a constant force to a single polyprotein composed of nine repeats of the small protein ubiquitin (*13*–*16*). This resulted in the probabilistic unfolding of ubiquitin, which was observed as stepwise elongations of the protein in which each step corresponded to the unfolding of an individual protein module (*12*). We applied this technique to monitor the end-to-end length of a single ubiquitin polyprotein (*17*) during reversible unfolding-folding cycles. Our experimental approach is illustrated in Fig. 1. Figure 1A shows the changes in the length of a single ubiquitin polyprotein in response to the stretching force displayed in Fig. 1B. As shown, stretching the polyubiquitin chain at 120 pN triggers a series of unfolding events that appear as a staircase of 20-nm steps, marking the unfolding of the individual ubiquitins in the chain (Fig. 1A). After 4 s, the force was relaxed to 15 pN (Fig. 1B) (*18*), and we observed the protein spontaneously contract in stages until it reached its folded length (Fig. 1A). To confirm that the polyprotein had folded, we raised the stretching force back to 120 pN at 14 s (Fig. 1B) and observed the ubiquitin chain extend in steps of 20 nm back to its fully unfolded length (Fig. 1A). Hence, the spontaneous contraction of the protein observed upon reducing the force from 120 pN down to 15 pN corresponds to the folding trajectory of the mechanically unfolded ubiquitin.

We observed and analyzed 81 folding events similar to those shown in Fig. 1. Two typical folding trajectories for mechanically unfolded polyubiquitin molecules are shown in Fig. 2. Most of the folding trajectories are qualitatively similar, following a continuous convex time course marked by abrupt changes in slope. However, we have never observed identical sets of trajectories, indicating the existence of multiple folding pathways for ubiquitin. To simplify the analysis of the folding trajectories, we divided their time course, roughly, into four distinct stages marked by abrupt changes in the slope of the collapse (Fig. 2). As an example, we analyze the recording shown in Fig. 2A. The first stage (1 in Fig. 2A and inset) is fast, lasting ~10 ms, which is slower than the time it takes the force to reach its set point (~3 ms in this experiment). The collapse rate for this stage (cr_1 = 2135 nm/s) is within the range but clearly slower than the maximum rate of change, or slew rate (*sr*), of the feedback during this experiment (measured at *sr* = ~8300 nm/s, after the molecule detached from the cantilever). This stage is likely to correspond to the elastic recoil of the unfolded polypeptide chain adjusting its length to the step change in the pulling force. This stage is always fast and is clearly marked in

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